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CONTROLLED DRUG DELIVERY FROM A NOVEL INJECTABLE *IN SITU* FORMED BIODEGRADABLE PLGA MICROSPHERE SYSTEM

Rajeev Jain
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CONTROLLED DRUG DELIVERY FROM A NOVEL
INJECTABLE *IN SITU* FORMED BIODEGRADABLE
PLGA MICROSPHERE SYSTEM

BY
RAJEEV JAIN

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
APPLIED PHARMACEUTICAL SCIENCES

UNIVERSITY OF RHODE ISLAND

1998

ABSTRACT

A novel method for *in situ* preparation of injectable biodegradable microspheres from the copolymer poly(lactide-*co*-glycolide) (PLGA) without incorporating unacceptable organic solvents is described. The delivery system is a dispersion of PLGA microglobules (“premicrospheres” or “embryonic microspheres”) in an acceptable vehicle mixture (continuous phase) and whose integrity is maintained by use of appropriate stabilizers. A solution of PLGA, triacetin, drug, PEG 400, and Tween 80 (Oil Phase 1) are added dropwise with continuous homogenization to miglyol 812-Span 80 solution (Oil Phase 2), thereby inducing phase separation (coacervation) of PLGA and forming PLGA microglobules (containing the drug) dispersed in the continuous phase. This novel drug delivery system (NDDS) is a dispersion and has a viscous consistency but is sufficiently syringeable. When injected, it comes in contact with water from aqueous buffer or physiological fluid and as a result, the microglobules harden to form solid matrix type microparticles entrapping the drug (*in situ* formed microspheres). The drug is then released from these microspheres in a controlled fashion.

This novel microencapsulation process overcomes some of the disadvantages associated with the existing methods by: (i) excluding the use of unacceptable organic solvents and using acceptable vehicle mixture instead to prepare biodegradable PLGA microspheres, (ii) forming drug containing PLGA microglobules (“premicrospheres” or “embryonic microspheres”) which could be considered as precursors to the final microsphere product; these on coming in contact with water harden to form discrete PLGA microspheres which subsequently exhibit non-variable, predictable, and controlled drug release profile, and (iii) precluding the need for reconstitution of the PLGA microspheres before their administration.

The composition, rationale, and optimization of the NDDS is described here. The characteristics of this NDDS were affected by various formulation

variables such as: (i) the PLGA concentration and type, (ii) the substitution of the continuous phase by a fresh Oil Phase 2, (iii) the concentration of PEG 400 and the encapsulated drug, (iv) the addition of an hydrophilic excipient (mannitol), and (v) the types of encapsulated drugs and the vehicles added to the system. The characteristics of the NDDS were reproducible and were not affected by a 15 days/4° C storage condition. Also, the formulation, process, and the storage (15 days/4° C) conditions did not adversely affect the physical stability of the encapsulated proteins.

Besides producing injectable *in situ* formed microspheres, this novel microencapsulation process can be modified to yield injectable *in situ* formed implant or isolated microspheres. Thus this novel microencapsulation process is versatile and it can produce various drug loaded injectable biodegradable PLGA devices having different characteristics.

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*To my wife Harsha for her love, encouragement, support, and everlasting
patience*

PREFACE

This dissertation has been written in the Manuscript Format as per the guidelines issued by The Graduate School at the University of Rhode Island. This option was most suitable to present my results in several sections.

Section I constitutes the Objectives and Introduction of this dissertation. The manuscripts in Section II are the core of this dissertation. Section III consists of the Conclusion and Final Remarks of this dissertation.

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LIST OF PUBLICATIONS

Manuscript I has been submitted for publication in the Drug Development and Industrial Pharmacy/Biotechnology Drug Development.

Manuscripts II, III, and IV will be submitted for publication in the Journal of Controlled Release, Pharmaceutical Research, and International Journal of Pharmaceutics respectively, pending patent submission.

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SECTION I

OBJECTIVES

The main intention of this research project were to achieve controlled drug delivery of micromolecules and macromolecules, such as proteins, from a novel injectable biodegradable poly(lactide-*co*-glycolide) (PLGA) microsphere system. This system would overcome some of the disadvantages associated with the traditional methods for controlled drug delivery. On injection, the system would come in contact with water from aqueous buffer or physiological fluid and as a result, form solid matrix type microparticles entrapping the drug (*in situ* formed microspheres); the drug would be released from these microspheres in a controlled fashion.

The specific objectives of this research project were as follows:

- (1) To develop a novel method for controlled delivery of drugs from an *in situ* forming biodegradable PLGA microsphere system.
- (2) To evaluate the effects of various formulation variables on the characteristics of this system.
- (3) To determine the effects of formulation, process and storage conditions on the reproducibility and stability of this system as well as the stability of the encapsulated proteins.
- (4) To modify this novel microencapsulation process, to produce *in situ* formed implant or isolated microspheres and also compare the characteristics of the three biodegradable devices: *in situ* formed implant v/s *in situ* formed microspheres v/s isolated microspheres.

INTRODUCTION

To avoid inconvenient surgical insertion of large implants, injectable biodegradable and biocompatible polymeric particles (microparticles and nanoparticles) could be employed for parenteral controlled-release dosage forms. Microparticles of size less than 250 μm , ideally less than 125 μm are suitable for this purpose. Biodegradable polymers are natural or synthetic in origin and are decomposed *in vivo*, either enzymatically or non-enzymatically to produce biocompatible, toxicologically safe by-products which are further eliminated by normal metabolic pathways. Drugs formulated in polymeric devices are released either by diffusion through the polymer barrier, or by erosion of the polymer material, or by a combination of both diffusion and erosion mechanisms. The polymers selected for the parenteral administration must meet several requirements like biocompatibility, drug compatibility, suitable biodegradation kinetics and mechanical properties, and ease of processing.

Although a wide variety of natural and synthetic biodegradable polymers have been investigated for drug targeting or prolonged drug release, only a few of them are actually biocompatible. Natural biodegradable polymers like bovine serum albumin (BSA), human serum albumin (HSA), collagen, gelatin, and hemoglobin have been studied for drug delivery. The use of these natural polymers is limited due to their higher costs and questionable purity.

In the past two decades synthetic biodegradable polymers have been increasingly used to deliver drugs, since they are free from most of the problems associated with natural polymers. Poly(amides), poly(amino acids), poly(alkyl- α -cyano acrylates), poly(esters), poly(orthoesters), poly(urethanes), and poly(acrylamides) have been used to prepare polymeric devices to deliver drugs. Amongst them, the thermoplastic aliphatic poly(esters) like poly(lactide) (PLA), poly(glycolide) (PGA), and especially the copolymer of lactide and glycolide referred to as poly(lactide-*co*-glycolide) (PLGA) have generated immense interest due to their excellent biocompatibility and biodegradability. Also PLGA has been

approved by the U.S. FDA for a number of clinical applications including surgical sutures and as controlled-release microspheres. PLGA is shown to be biocompatible and degrades to toxicologically acceptable lactic and glycolic acids that are eventually eliminated from the body. Release of drugs from PLGA microspheres occurs by two mechanisms: (i) diffusion of the drug through a tortuous, water-filled path in the polymer matrix and (ii) matrix bioerosion (bulk hydrolytic degradation) after undergoing sufficient hydration. The actual release is a combination of both the processes.

There is a particular interest in controlled delivery of macromolecules like peptides and proteins through PLGA microspheres. Although a wide variety of pharmacologically useful peptide and protein based drugs have been recently developed by genetic engineering, their therapeutic use is restricted due to certain disadvantages: (i) on oral consumption they are subject to attack by the acidic and enzymatic environment in the stomach and the enzymes from the brush border membrane of the intestine, (ii) their high molecular weight and size impede their effective transport across the gastrointestinal membranes, and (iii) they have a short biological half-life and on injection they are quickly metabolized and eliminated. To achieve sustained blood levels of these drugs, minimize their denaturation or degradation, and to extend their biological half-life, their delivery by encapsulation in PLGA microspheres has become an interesting approach.

The literature on PLGA microspheres is full of different techniques describing their manufacture, where the microspheres are produced in a free-flowing, powder form. Some of the methods reported are: (i) single/double emulsification followed by solvent removal by evaporation or extraction, (ii) phase separation (coacervation), and (iii) spray-drying. Most of these manufacturing processes suffer from drawbacks such as: (i) the microspheres need to be reconstituted (suspended) in an aqueous media, before they could be injected in the body, (ii) the hazards and environmental concern associated with the use of organic solvents like methylene chloride for the solubilization of PLGA polymer, and (iii) residual organic solvents remaining in the final microsphere product.

Shah *et al* and researchers from Atrix Laboratories, Inc. (Fort Collins, CO) have described a novel implant system which is parenterally administered as a liquid and subsequently solidifies into a gel matrix (implant) *in situ*, from which the drug is released in a controlled manner. Although this implant system precludes the need for any surgery for its administration, it has a number of disadvantages: (i) the safety of solvents like N-methyl-2-pyrrolidone, used to formulate these systems is questionable and not well documented, (ii) the injection of these liquid implant systems and their subsequent solidification produce non-uniform matrix implants having variable consistency and geometry, and (iii) due to formation of matrix implants having inconsistent texture, shape and size, the drug release from them is variable and unpredictable.

The present process of microsphere formation is based on the principle of coacervation. This method overcomes the problems faced by the above systems by forming a dispersion of PLGA microglobules ("premicrospheres" or "embryonic microspheres") in an acceptable vehicle mixture (continuous phase) and whose integrity is maintained by use of appropriate stabilizers. A solution of PLGA, triacetin, drug, PEG 400, and Tween 80 (Oil Phase 1) are added dropwise with continuous homogenization to miglyol 812-Span 80 solution (Oil Phase 2), thereby inducing phase separation (coacervation) of PLGA and forming PLGA microglobules (containing the drug) dispersed in the continuous phase. This novel drug delivery system (NDDS) is a dispersion and has a viscous consistency, but is sufficiently syringeable. When injected, it comes in contact with water from aqueous buffer or physiological fluid and as a result, the microglobules harden to form solid matrix type microparticles entrapping the drug (*in situ* formed microspheres). The drug is then released from these microspheres in a controlled fashion. This novel microencapsulation method can be modified to produce other biodegradable PLGA devices exhibiting controlled drug delivery.

SECTION II

MANUSCRIPT I

CONTROLLED DRUG DELIVERY BY BIODEGRADABLE POLY(ESTER)
DEVICES: DIFFERENT PREPARATIVE APPROACHES

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ABSTRACT

There has been extensive research on drug delivery by biodegradable polymeric devices since bioresorbable surgical sutures entered the market two decades ago. Amongst the different classes of biodegradable polymers, the thermoplastic aliphatic poly(esters) like poly(lactide) (PLA), poly(glycolide) (PGA), and especially the copolymer of lactide and glycolide referred to as poly(lactide-co-glycolide) (PLGA) have generated interest due to their excellent biocompatibility, biodegradability, and mechanical strength. Also, they are easy to formulate into various devices for carrying a variety of drug classes such as vaccines, peptides, proteins, and micromolecules; most importantly they have been approved by the Food and Drug Administration (FDA) for drug delivery.

This review presents different techniques of preparation of various drug loaded PLGA devices, with special emphasis on preparing microparticles. Certain issues about other related biodegradable polyesters are discussed.

INTRODUCTION

A controlled drug action may be achieved by either chemically modifying the drug moiety (e.g. prodrug) or by formulating it in a specific way to control its release. Oral controlled-release dosage forms, depending upon the drug employed, can provide efficacy for about 24 hr. (1). Oral dosage forms may not be feasible in cases where the drug undergoes extensive degradation in the gastrointestinal tract, exhibits significant first-pass effect, or is poorly absorbed. Of serious concern are the problems associated with the oral administration of peptide/protein drugs which are subject to attack by the acidic and enzymatic environment in the stomach and the enzymes from the brush border membrane of the intestine. Also their high molecular weight and size impede their effective transportation across the gastrointestinal tract membranes. The main drawback of oral dosage forms are the short transit time of approximately twelve hours through the gastrointestinal tract (2). Further, if the drug is absorbed only through a specific area of the gastrointestinal tract, the duration of action could be less than twelve hours (2).

If a drug cannot be administered orally due to any of the above reasons, a parenteral route of delivery is an alternative. One advantage that a parenteral controlled release dosage form has over oral controlled release dosage forms is patient compliance (2). Although an oral dosage form might have a good bioavailability, a long-acting parenteral dosage form that is safe and efficacious for days or weeks or months could be beneficial because it ensures that the patient is receiving medication. Also a parenteral controlled release dosage form is preferred over conventional parenteral dosage form for chronic treatment where routine multiple injections could be inconvenient and painful. Parenteral controlled release dosage forms are also effective in site-specific drug delivery, thereby improving its efficacy and reducing its toxicity. The main disadvantage of these dosage forms is that once administered, they cannot be easily removed (2). This could be a problem for the patient if a drug was no longer needed, or worse if it caused an undesirable reaction.

To avoid inconvenient surgical insertion of large implants, injectable biodegradable and biocompatible polymeric particles (microspheres, microcapsules, nanocapsules, nanospheres) could be employed for parenteral controlled-release dosage forms (1). Microparticles of size less than 250 μm , ideally less than 125 μm are suitable for this purpose (2). Biodegradable polymers are natural or synthetic in origin and are decomposed *in vivo*, either enzymatically or non-enzymatically to produce biocompatible, toxicologically safe by-products which are further eliminated by normal metabolic pathways (3). Drugs formulated in polymeric devices are released either by diffusion through the polymer barrier, or by erosion of the polymer material, or by a combination of both diffusion and erosion mechanisms (4). The polymers selected for the parenteral administration must meet several requirements like biocompatibility, drug compatibility, suitable biodegradation kinetics and mechanical properties, and ease of processing (4, 5).

Although a wide variety of natural and synthetic biodegradable polymers have been investigated for drug targeting or prolonged drug release, only a few of them are actually biocompatible. Natural biodegradable polymers like bovine serum albumin (BSA), human serum albumin (HSA), collagen, gelatin, and hemoglobin have been studied for drug delivery (1). The use of these natural polymers is limited due to their higher costs and questionable purity (1).

In the past two decades synthetic biodegradable polymers have been increasingly used to deliver drugs, since they are free from most of the problems associated with natural polymers (1-8). Poly(amides), poly(amino acids), poly(alkyl- α -cyano acrylates), poly(esters), poly(orthoesters), poly(urethanes), and poly(acrylamides) have been used to prepare polymeric devices to deliver drugs (1-7). Amongst them, the thermoplastic aliphatic poly(esters) like poly(lactide) (PLA), poly(glycolide) (PGA), and especially the copolymer of lactide and glycolide referred to as poly(lactide-co-glycolide) (PLGA) have generated immense interest due to their excellent biocompatibility and

biodegradability (1-17). Also they are easy to formulate into drug carrying devices and have been approved by the FDA for drug delivery use (13-17).

This review provides a comprehensive outlook on different techniques of preparation of various drug loaded PLGA devices, with special emphasis on preparing microparticles. Certain issues about other related biodegradable polyesters like PLA and PGA have been discussed as well.

HISTORICAL DEVELOPMENT OF DRUG DELIVERY USING PLGA

The discovery and the synthetic work on low molecular weight oligomeric forms of lactide and/or glycolide polymers was first carried out several decades back (3, 5). The methods to synthesize high molecular weights of these polymers were first reported by Lowe (3).

During the late 1960s and early 1970s a number of groups had published pioneering work on the utility of these polymers to make sutures/fibers (2, 3, 5, 12). These fibers had several advantages such as good mechanical properties, low immunogenicity and toxicity, excellent biocompatibility, and predictable biodegradation kinetics (2, 3, 5, 12). The wide acceptance of the lactide/glycolide polymers as suture materials, made them an attractive candidate for biomedical applications like ligament reconstruction, tracheal replacement, ventral herniorrhaphy, surgical dressings, vascular grafts, nerve, dental, and fracture repairs (3, 5, 9).

The biodegradation, biocompatibility, and tissue reaction of PLA and PLGA have been extensively investigated and well documented by many researchers (5, 14). The first work on parenteral controlled release of drugs using PLA was reported by Boswell, Yolles, Sinclair, Wise, and Beck (3, 5). Since then an ocean of literature on drug delivery using PLA, and especially PLGA has been published. Various polymeric devices like microspheres, microcapsules, nanoparticles, pellets, implants, and films have been fabricated using these polymers for the delivery of a variety of drug classes.

SYNTHESIS OF PLGA COPOLYMER

Low molecular weight PLGA can be prepared by direct condensation (polyesterification) of lactic and/or glycolic acids (5, 12). Temperatures as high as 130-190° C are required for the condensation process and the water generated is removed by boiling, using vacuum, purging with nitrogen, or azeotropic distillation with an organic solvent (3, 12). An acid catalyst like antimony oxide increases the reaction rate if used at reaction temperatures below 120° C, but above this temperature water removal is the rate-limiting step (3, 12). This method yields PLGA having molecular weight of ~10,000 (12). The low molecular weight PLGA has limited biomedical application, due to its poor mechanical strength and faster degradation (3).

Intermediate and high molecular weight PLGA (~10,000-40,000) can be prepared by the ring-opening polymerization of the cyclic dimers (cyclic diester of lactic and/or glycolic acids) as the starting materials (3, 5, 12, 14). The advantage of this method is that no water removal/dehydration method is needed in the polymerization system (3). Also the cyclized monomer(s) and the linear form of the polymers produced can be readily purified (3). Compounds of lead, tin, cadmium, zinc, antimony, and titanium have been used as catalyst to initiate the polymerization process (12, 14). Acid catalyzed bulk polymerization (melt method) for two to six hours at around 175° C is generally employed for preparation of PLGA from lactide and glycolide monomers (3). The molecular weight of the resultant PLGA is determined by the concentration of the catalyst added (12). Monomer purity of 99.9% or greater and monomer acidity of 0.05% or less are required with the starting lactide and glycolide materials (5). Also important are the low levels of humidity in the processing area (5).

PHYSICAL, CHEMICAL, AND BIOLOGICAL PROPERTIES OF PLGA

It is important to understand the physical, chemical, and biological properties of the polymer before formulating a controlled drug delivery device. The various properties of the polymer and the encapsulated drug directly

influence other factors like the selection of the microencapsulation process, drug release from the polymer device, etc. (1).

PLA can exist as the optically active stereoregular polymer (L-PLA) and an optically inactive racemic polymer (D, L-PLA) (1, 5, 9). L-PLA is found to be semicrystalline in nature due to high regularity of its polymer chain while D, L-PLA is an amorphous polymer because of irregularities in its polymer chain structure (3, 9). Hence the use of D, L-PLA is preferred over L-PLA as it enables more homogeneous dispersion of the drug in the polymer matrix (9, 13). PGA is highly crystalline because it lacks the methyl side groups of the PLA (3, 9). Lactic acid is more hydrophobic than glycolic acid and hence lactide-rich PLGA copolymers are less hydrophilic, absorb less water, and subsequently degrade more slowly (1, 3, 13).

The molecular weight and polydispersity index of the polymer are factors which affect the mechanical strength of the polymer and its ability to be formulated as a drug delivery device (3, 5, 12). Also these properties may control the polymer biodegradation rate and hydrolysis (3, 12). The commercially available PLGA polymers are usually characterized in terms of intrinsic viscosity which is directly related to their molecular weights (3).

The degree of crystallinity of the PLGA polymer directly influences its mechanical strength, swelling behavior, capacity to undergo hydrolysis, and subsequently its biodegradation rate (3). The resultant crystallinity of the PLGA copolymer is dependent on the type and the molar ratio of the individual monomer components (lactide and glycolide) in the copolymer chain (1). PLGA polymers containing 50:50 ratio of lactic and glycolic acids are hydrolyzed much faster than those containing higher proportion of either of the two monomers (5, 12). PLGAs prepared from L-PLA and PGA are crystalline copolymers while those from D, L-PLA and PGA are amorphous in nature (3, 5). Gilding and Reed have pointed out that PLGAs containing less than 70 % glycolide are amorphous in nature (18). The degree of crystallinity and the melting point of the polymers are directly related to the molecular weight of the polymer (3, 5).

The glass transition temperature (T_g) of the PLGA copolymers are above the physiological temperature of 37° C and hence they are glassy in nature (3, 5). Thus they have a fairly rigid chain structure which gives them significant mechanical strength to be formulated as drug delivery devices (3, 5). Jamshidi *et al.* have reported that T_g of PLGAs decrease with decrease of lactide content in the copolymer composition and with decrease in their molecular weight (19).

It is important for the PLGA polymers to have considerable mechanical strength since the drug delivery devices formulated using them are subjected to significant physical stress (3, 5). Different factors like the molecular weight, copolymer composition (lactide/glycolide ratio), crystallinity, and geometric regularity of individual chains significantly affect the mechanical strength of the polymer (1, 3, 5).

In vitro and *in vivo* the PLGA copolymer undergoes degradation in an aqueous environment (hydrolytic degradation or biodegradation) through cleavage of its backbone ester linkages (1-3, 5, 12, 13). The polymer chains undergo bulk degradation and the degradation occurs at uniform rate throughout the PLGA matrix (3, 13). Thies and Bissery have reported that the PLGA biodegradation occurs through random hydrolytic chain scissions of the swollen polymer (20). The carboxylic end groups present in the PLGA chains increase in number during the biodegradation process as the individual polymer chains are cleaved; these are known to catalyze the biodegradation process (3, 5). The biodegradation rate of the PLGA copolymers are dependent on the molar ratio of the lactic and glycolic acids in the polymer chain, molecular weight of the polymer, the degree of crystallinity, and the T_g of the polymer (3, 5, 13). A three phase mechanism for the PLGA biodegradation has been proposed (21):

1. Random chain scission process. The molecular weight of the polymer decreases significantly, but no appreciable weight loss and no soluble monomer products formed.
2. In the middle phase a decrease in molecular weight accompanied by rapid loss of mass and soluble oligomeric and monomer products are formed.

3. Soluble monomer products formed from soluble oligomeric fragments. This phase is that of complete polymer solubilization.

The extent, if any, on the role of enzymes in the PLGA biodegradation is unclear (3, 5). Most of the literature indicates that the PLGA biodegradation does not involve any enzymatic activity and is purely through hydrolysis (3). However, some investigators have suggested an enzymatic role in PLGA breakdown based upon the difference in the *in vitro* and *in vivo* degradation rates (5).

The PLGA polymer biodegrades into lactic and glycolic acids (1-3, 5, 12, 13). Lactic acid enters the tricarboxylic acid cycle and is metabolized and subsequently eliminated from the body as carbon dioxide and water (1-3, 5, 9). In a study conducted using ^{14}C -labeled PLA implant, it was concluded that lactic acid is eliminated through respiration as carbon dioxide (22). Glycolic acid is either excreted unchanged in the kidney or it enters the tricarboxylic acid cycle and is eventually eliminated as carbon dioxide and water (3).

METHODS OF PREPARING VARIOUS PLGA DEVICES

[1] MICROPARTICLES

A number of microencapsulation techniques have been developed and reported to date. The choice of the technique depends on the nature of the polymer, the drug, the intended use, and the duration of the therapy (1, 2, 4, 5, 10). The microencapsulation method employed must include the following requirements (1, 2, 23):

- (i) The stability and biological activity of the drug should not be adversely affected during the encapsulation process or in the final microsphere product.
- (ii) The yield of the microspheres having the required size range (upto 250 μm , ideally < 125 μm) and the drug encapsulation efficiency should be high.
- (iii) The microsphere quality and the drug release profile should be reproducible within specified limits.

(iv) The microspheres should be produced as a free flowing powder and should not exhibit aggregation or adherence.

A. Solvent Evaporation and Solvent Extraction Process

(1) Single emulsion process

This is essentially an oil-in-water (o/w) emulsion process. The polymer is first dissolved in a water immiscible, volatile organic solvent; dichloromethane (DCM) most commonly used. The drug is then added to the polymer solution to produce a solution or dispersion of the drug particles (particle size of the drug added to be $< 20\ \mu\text{m}$) (4). This polymer-solvent-drug solution/dispersion is then emulsified (with appropriate stirring and temperature conditions) in a larger volume of water in presence of an emulsifier (such as poly (vinyl alcohol) (PVA)) to yield an o/w emulsion. The emulsion is then subjected to solvent removal by either evaporation or extraction process to harden the oil droplets (10). In the former case the emulsion is maintained at reduced pressure or at atmospheric pressure and the stirring rate reduced to enable the volatile solvent to evaporate (4, 10). In the latter case the emulsion is transferred to a large quantity of water (with or without surfactant) or other quench medium, into which the solvent associated with the oil droplets diffuses (4, 10). The solid microspheres so obtained are then washed and collected by filtration, sieving, or centrifugation (4). These are then dried under appropriate conditions or are lyophilized to give the final free flowing injectable microsphere product.

It should be noted that the solvent evaporation process in a way is similar to the extraction method, in the sense that the solvent must first diffuse out into the external aqueous dispersion medium before it could be removed from the system by evaporation (4, 10). The rate of solvent removal by the extraction method depends on the temperature of quench water or other medium, ratio of emulsion volume to quench water/medium volume and the solubility characteristics of the polymer, the solvent, and the dispersion medium. The rate of solvent removal by evaporation method strongly influences the characteristics of the final microspheres and it depends on the temperature, pressure, and the

solubility parameters of the polymer, the solvent, and the dispersion medium (10). Very rapid solvent evaporation may cause local explosion inside the droplets and lead to formation of porous structures on the microsphere surface (10). Solvent removal by extraction method is more faster (generally < 30 min) than the evaporation process and hence the microspheres made by the former method are more porous in comparison to those made from the latter method under similar conditions (10).

The biggest drawback of the o/w emulsification method is poor encapsulation efficiencies of moderately water soluble and water soluble drugs (1, 4, 10). The drug would diffuse out or partition from the dispersed oil phase into the aqueous continuous phase and microcrystalline fragments of the hydrophilic drugs get deposited on the microsphere surface and dispersed in the PLGA matrix (24, 25). This would result in poor trapping of the hydrophilic drug such as salicylic acid and initial rapid release of the drug (burst effect) (1). The o/w emulsification process is therefore widely used to encapsulate lipid soluble drugs like steroids (1).

To increase the drug loading of water soluble drugs, an oil-in-oil (o/o) emulsification method was developed (1, 10, 26). A water-miscible organic solvent like acetonitrile is employed to solubilize the drug in which PLGA or PLA are also soluble. This solution is then dispersed into an oil such as light mineral oil in presence of an oil soluble surfactant like Span to yield the o/o emulsion. Microspheres are finally obtained by evaporation or extraction of the organic solvent from the dispersed oil droplets and the oil is washed off by solvents like n-hexane. This process is also sometimes referred as water-in-oil (w/o) emulsification method (1).

A number of formulation and process factors affect microsphere formation. The main variables that influence the microencapsulation process and the final microsphere product are: (a) the nature and solubility of the drug being encapsulated; (b) the polymer concentration, composition, and molecular weight; (c) the drug/polymer ratio; (d) the organic solvent used; (e) the concentration and

nature of the emulsifier used; (f) the temperature and stirring/agitation speed of the emulsification process; and (g) the viscosities and volume ratio of the dispersed and continuous phases (1, 4, 5, 10).

(i) Solvents

Selection of dispersed and continuous phase is important for successful microsphere formation and to achieve high drug encapsulation efficiencies. For the solvent evaporation/extraction method the dispersed phase selected should be immiscible or only slightly miscible with the continuous phase and must have a boiling point lower than that of the continuous phase (4). Bodmeier and McGinity have shown that water miscible solvents like acetone and dimethyl sulfoxide do not form microspheres upon emulsification (27). Typically, DCM and water are used as dispersed and continuous phases respectively. DCM is widely used because it is a good solvent for the polymers and due to its high volatility it can be easily removed by evaporation.

A major problem with the use of DCM is its potential toxicity (28). Chlorinated solvents in general are considered hazardous to environment and undesirable for use in manufacturing processes (28). Chern et al. have reported the use of ethyl acetate to prepare PLGA microspheres by the solvent extraction process (29). Sah *et al.* produced microspheres by a two-step extraction process using methyl ethyl ketone (MEK) (10 times more soluble than DCM in water) as the solvent for PLGA (28, 30). Rapid diffusion of MEK into the extraction medium and migration of water into the oil droplets produced hollow microspheres having volume mean diameter of 96 μm and 60 to 77% drug entrapment (28, 30). The authors concluded that water-immiscibility of the dispersed phase is not an absolute requirement for the solvent evaporation/extraction process (28, 30).

For o/o emulsification method, acetonitrile (26, 31-38) is generally used as the dispersed phase. Other solvents like acetonitrile/water mixture (24), DCM (39), N,N-dimethyl formamide (DMF) (40-42) have also been used. The continuous phase consists of oils like light mineral oil (26, 33-36, 38, 39), cotton

seed oil (24), liquid paraffin (40-42), silicon oil (31), and machine oil (37). When prepared by solvent extraction method, heptane has been most commonly used as extraction medium (32-38). Thanoo *et al.* prepared microspheres from PLA using DCM, glycerin, and isopropanol/water mixture as the dispersed phase, continuous phase, and extraction medium respectively (43). The same group also reported preparation of PGA microspheres using hexafluoroacetone, carbon tetrachloride, and dioxan as the dispersed phase, continuous phase, and extraction medium respectively (43).

In an article van Hamont *et al.* have concluded that the particle size of the microspheres is a balance of the following two opposite actions: (a) higher weights of the external oil phase tend to produce larger diameter microspheres due to slowing of the solvent evaporation process and (b) decrease in polymeric droplet coalescence due to increase in viscosity of the oil phase tend to decrease the diameter of the microspheres (37).

Sometimes a solvent mixture rather than a solvent alone is employed as the dispersed phase (43). Such a solvent mixture consists of a water-immiscible solvent such as DCM (44, 45) or chloroform (46, 47) and a water-miscible solvent like acetone (44, 46, 47), methanol (45), ethanol (4), or propylene glycol (4). The water-miscible solvent provides rapid solvent removal and faster polymer precipitation and hardening (44-47). Coombes *et al.* used DCM-acetone mixture in the solvent evaporation process and concluded that solvent removal process is rapid and causes entrapment of the stabilizer molecules by physical chain entanglement and thus enhancing their stabilizing capacity (44). Use of DCM alone, however, results in a slow solvent evaporation process, allowing entrapped stabilizer molecules to diffuse out into the external aqueous phase with consequent loss of their stabilizing capacity (44). Thanoo *et al.* have prepared PLGA microspheres using a mixture of two water-immiscible solvents, DCM and chloroform by the solvent evaporation process (43). Polard *et al.* have reported that due to poor solubility of morphine in DCM, and good solubility in methanol, methanol was used as hydrophilic cosolvent (45). As the fraction of methanol

was increased in the DCM/methanol mixture, more morphine dissolved in the organic phase and this enhanced the drug entrapment in the microspheres as a result of faster precipitation of the polymer (45). However when percentage of methanol in the solvent mixture exceeded 60%, the polymer could not be dissolved (45). Spenlehauer *et al.* employed DCM:cyclohexane (10:1) mixture for producing PLA microspheres (48). Cyclohexane is less volatile than DCM and hence evaporation of DCM from the emulsion droplets leads to entrapment of cyclohexane in the microspheres, resulting in formation of porous surface structures during the final removal of cyclohexane (48).

Sansdrap and Moës have found that the increase in the external aqueous phase volume did not affect the final microsphere size while increase in the dispersed DCM volume decreased the size with narrow size distribution due to the decrease in the viscosity of the internal phase with increasing volume (49).

(ii) Emulsifiers

During the solvent evaporation/extraction process, there is a gradual decrease in the volume and subsequent increase in the viscosity of the dispersed oil droplets (4). This affects the droplet size equilibrium and the droplets tend to coalesce and produce agglomerates during the early stages of solvent removal (1, 4, 10). This problem could be rectified by adding a small quantity of a droplet stabilizer (emulsifier) in the continuous phase (1, 4, 10). The emulsifier provides a thin protective layer around the oil droplets, and hence reduces their coalescence and coagulation (10). As the solvent is removed, the emulsifier continues to maintain the spherical shape of the oil droplets and prevents their aggregation, until the microspheres are hardened and isolated as discrete particles (4).

The physicochemical properties and the concentration of the emulsifier strongly influences the microsphere size, shape, and drug encapsulation efficiency. The emulsifiers most commonly used in the solvent evaporation/extraction process are the hydrophilic polymeric colloids and/or anionic or nonionic surfactants (4, 10). PVA is by far the most commonly used emulsifier (25, 29, 30, 33-35, 45-47, 50-63) in the o/w emulsion method. Others

include poly vinyl pyrrolidone (PVP) (4), alginate (4), gelatin (4), methyl cellulose (MC) (25, 51, 54, 64, 65), hydroxyalkyl cellulose (4, 10), Hydroxypropylmethyl cellulose (HPMC) (49), polyoxyethylene derivatives of sorbitan fatty esters (Tweens) (4, 10), cetyltrimethyl ammonium bromide (4, 10), and fatty acid salts like sodium oleate (43, 66-68). For o/o method, oil soluble emulsifiers such as polyoxyethylene fatty ethers (Brijs), Spans, and lecithins have been used (1, 4, 10).

The appropriate type and concentration of the emulsifier for a particular process is apparently commonly determined by trial and error basis, although optimization techniques clearly have potential in this area. For most of the emulsifiers, the microsphere size decreases with increase in emulsifier concentration (4, 49). Beyond a certain concentration, the emulsifier is ineffective, due to achievement of an optimal packing concentration for the emulsion, i.e. condensed monolayer (40). Wakiyama *et al.* have investigated the emulsifying action of sodium alginate in comparison with gelatin and have concluded that sodium alginate produced a relatively more viscous aqueous phase and hence yielded relatively smaller microspheres as compared to those produced by the same amount of gelatin (69). Fong *et al.* found that when sodium hydroxide was added to the aqueous continuous phase, the ionization of the emulsifier sodium oleate was increased, which resulted in higher drug encapsulation efficiencies and smaller, spherical, but highly porous microspheres (70). Jalil and Nixon studied the effects of oil soluble emulsifiers (Spans and Brijs) on the size of microspheres prepared by o/o emulsion and concluded that more hydrophilic emulsifiers produced smaller microspheres (71). Coombs *et al.* prepared PLGA microspheres using various grades of poly(oxyethylene)-poly(oxypropylene) (PEO-PPO) co-polymers as the surfactants (44). The solvent removal led to entrapment of these surfactant molecules by physical chain entanglement and their location at the microsphere surface. The authors stated that the PEO-PPO chain length, structure, and conformation influenced the

surface coverage of the microspheres, the strength of surfactant attachment, and its overall performance (44).

Some times a combination of emulsifiers have been used to achieve the necessary emulsifying action (25, 51, 72). Cavalier *et al.* have reported that a combination of PVA and MC yielded PLA microspheres having maximum sphericity and drug entrapment as compared to formulations that used these individual colloids alone (25). This was due to improvement in the rheological properties of the combined emulsifiers as compared to their properties when used alone. A similar finding was reported by Spenlehauer *et al.* When the theoretical drug loading ranged from 0-30%, 0.25% aqueous PVA solution gave microspheres in the size range 25-50 μ m (51). However, for drug loading in the range of 50-60%, 0.25% MC or a PVA:MC (50:50) mixture was necessary to produce the microspheres (51).

(iii) Polymer

The polymer type, its molecular weight, and the concentration used strongly influence the characteristics of the final microspheres. Cavalier *et al.* have reported that a decrease in PLA concentration (increase in drug/PLA ratio) resulted in higher drug content in the microspheres (25). The same group also reported slightly higher drug content for PLGA (65:35) microspheres against those for PLA microspheres (25). Coombes *et al.* have reported a decrease in polydispersity and particle size of the microparticles as the PLGA concentration in it was decreased (44). In a study, drug content of PLA (molecular weight 2000) microparticles was higher than PLGA (molecular weights 9000 and 12000) and PLA (molecular weight 9000) microparticles due to the rapid rate of polymer precipitation at the droplet surface (45). The particle size increased from 1.0 μ m for PLGA (RG 505), to 1.1 μ m for PLGA (RG 858), to 1.5 mm for PLA (R 208) microspheres (73). The drug entrapment was however same for RG 505 and R 208 (2.8% w/w) while for RG 858 it was slightly higher (3.2% w/w) (73). In another study, microspheres prepared from 16% w/w PLGA had many structural defects while those prepared from 5.3% w/w had little structural defects but were

aggregated and formed lumps (40). In spite of an increase in the PLGA molecular weight from 6600 to 19000, microspheres with uniform particle size and no structural defects were produced (40). Udupa and Chandrashekar have reported a decrease in drug content and increase in microsphere size with increase in PLGA/drug ratio. In a peptide adsorption study, Calis *et al.* found that with increase in microsphere concentration (and hence PLGA concentration) the time for maximum peptide adsorption decreased (66). Delgado *et al.* have reported that values of certain polymer parameters like polydispersity and degradation index (a measure of polymer erosion) are directly related to the weight average molecular weight (M_w) of the PLA polymer used for microencapsulation (61).

(iv) Drugs

The biggest disadvantage of the o/w emulsification method is poor encapsulation of water soluble drugs (1, 4, 10). The o/w emulsification process is therefore recommended to encapsulate lipid soluble drugs (1). Several investigators have tried various modifications of the o/w method to minimize partitioning and thereby increase the entrapment of water soluble drugs (74 and more). Bodmeier and McGinity achieved higher entrapment of ionizable drugs like diazepam and quinidine by using high pH external aqueous phase (pH 12), where the loss due to ionization of these drugs was reduced (74). Similarly, Wakiyama reported higher drug encapsulation efficiencies for butamben and dibucaine when the aqueous phase consisted of 1% alkali (high pH solution) (69). However tetracaine under similar conditions got ionized and exhibited poor drug entrapment (69). Polard *et al.* used an external phase having a pH of 9 to prevent the solubility of morphine in water and thereby reducing its partitioning in the external aqueous phase (45). Contrary to these results, Vaughan *et al.* have reported that increasing the pH of the external aqueous phase to 10, did not increase the loading efficiency of lidocaine (33).

The loss of drug can also be minimized by presaturating the aqueous or organic phase with the same drug. The drug content of quinidine in PLA microspheres increased with increase in quinidine content in the dispersed organic

phase (74), while tetracaine entrapment increased with prior saturation of the aqueous phase (69).

Sah *et al.* have reported that the encapsulation efficiency of PLGA microspheres decreased with increasing theoretical loading of the drug (progesterone) due to rapid partitioning of the drug in the external aqueous phase from the dispersed organic phase (which contained MEK) (30). Polard *et al.* have shown that with increase in drug loading, the drug content of the microspheres increased but their encapsulation efficiencies and the microsphere recovery yield decreased (45). Also drug entrapment was higher when the drug was present in suspension form as compared to when present in the solution form (45). A similar result was reported by Cavalier *et al.*, where an increase in the drug/polymer ratio resulted in increase in the drug content of the microspheres (25). Thanoo *et al.* have shown an increase in the drug incorporation efficiency and the microsphere yield with increase in the theoretical drug loading (43).

Rosilio *et al.* have reported that for progesterone loading of 0-30%, the microsphere (prepared by o/w method) size was in the range 25-50 μm , and for 35% loading it increased to 50-75 μm (51). A different observation was made by Tsai *et al.* who prepared microspheres by o/o method (26). In spite an increase in the drug loading from 3.65 to 13.80 %, the microspheres exhibited an average size of 95 μm , with a relatively narrow size distribution (26). In another study, an increase in nifedipine (a water insoluble drug) loading resulted in subsequent increase in its content in the PLGA microspheres but did not influence the mean particle size (49).

Calis *et al.* carried out peptide adsorption studies and concluded that in dilute peptide solutions, peptide-PLGA interaction favored monolayer adsorption which fitted the Langmuir adsorption, while at higher peptide concentration, peptide-peptide interaction are favored, resulting in multilayer adsorption which fitted the Freundlich model (66). In another study, Duggirala *et al.* have showed that with increased protein loading, the adsorption of protein on PLGA

microspheres increased upto a definite value and then remained constant due to saturation of the microsphere surface (monolayer coverage) by the protein (75).

Bodmeier and McGinity have shown by a Scanning Electron Microscopy (SEM) study that for PLGA microspheres, the surface changed from a smooth texture at low drug content to a porous honey-comb like structure at higher drug loading (74). In another study, PLGA microcapsules containing 8% progesterone showed a smooth external morphology while those containing 21% drug exhibited textured and irregularly shaped surface features (30). When the theoretical progesterone loading was increased from 10 to 50%, the microsphere surface changed from a smooth, uniform appearance to an irregular surface containing well-defined progesterone crystals and numerous pores (51).

In a study carried out by Benoit *et al.*, increase in the encapsulated drug amount resulted in a gradual decrease in the Tg of PLGA polymer from 48.3 to 12.9° C (52). The authors concluded that the drug was molecularly dispersed in an amorphous form in PLGA (formation of a stable solution) and thus strongly plasticized the polymer (52). A similar interaction phenomena between the drug and PLGA has been reported by Crossan and Whateley (53) and Richey and Harris (76). Rosilio *et al.* have concluded that below 35% loading, progesterone is molecularly dispersed in the PLGA glass (51). At 35% and above, crystal domains of the steroid appeared and two crystalline forms, α and β could be detected (51). Bodmeier and McGinity (74) and Cavalier *et al.* (25) have also reported similar results of a molecular dispersion of the drug in the polymer glass.

(v) Process

Sah *et al.* prepared microspheres by a two-step extraction-hardening process using MEK as a solvent for the PLGA polymer (external aqueous phase was presaturated with MEK) (28, 30). In the first step, the emulsion was transferred into 250 ml of aqueous PVA solution where MEK was extracted out (30). In the next step the microcapsules were transferred into 500 ml of aqueous PVA solution for complete hardening of the microcapsules. The authors concluded that the initial extraction rate of MEK were critical for successful

microencapsulation (30). Also, the particle size of the microspheres decreased when increasing amount of MEK was predissolved in the external aqueous phase before the emulsification process (28). Giordanao *et al.* used DCM saturated 1% PVA aqueous phase to make PLGA microspheres (56). Rosilio *et al.* prepared microspheres from PLGA where the solvent (DCM) was removed by an interrupted process (51). DCM evaporation was interrupted after a definite period and the aqueous phase (continuous phase) was completely removed by several decantation washings. The DCM evaporation was then continued until the microspheres were obtained. This method was developed to minimize formation of emulsifier-assisted drug crystals at the microsphere surface and to achieve higher drug loading (51). Cowsar *et al.* produced microspheres from PLGA by two techniques: solvent extraction-solvent evaporation and solvent evaporation-solvent extraction (47). In the former case, most of the acetone was first allowed to diffuse out from the dispersed organic phase (chloroform-acetone mixture) into the external aqueous phase, followed by gradual evaporation of the residual solvents to give the final microspheres. In the latter case, the o/w emulsion was first subjected to solvent (DCM) evaporation for a certain period until semisolid droplets were obtained and the residual DCM was removed by the extraction process in a large volume of water. Microspheres from evaporation-extraction process were less porous and exhibited better encapsulation than those prepared from extraction-evaporation process (47).

Vaughan *et al.* (33, 34) and Pak *et al.* (35) have compared the effects of the solvent extraction v/s evaporation process on the final microsphere product. Microencapsulation of lidocaine base by the evaporation process gave product with an yield of 65-80%, volume mean diameter of 120-130 μm , drug content of 4-10%, smooth and non-porous surface, and only 30-70% loading efficiency (due to solubility of lidocaine in the external aqueous phase) (33). The extraction process, however yielded microspheres having lidocaine content in the range of 5-20%, particle size of 7-10 μm , smooth but very porous particles, and 100% loading efficiencies (33). The authors had used the salt form of the drug

(lidocaine hydrochloride), and not lidocaine base for the extraction process (33). Extraction process using lidocaine base resulted in encapsulation efficiency of less than 10% (33). The same group also reported a better product from the extraction process for the drug ketoprofen in terms of drug content, loading efficiency, particle size, and surface feature as against the evaporation process (34). Contrary to these results, Pak *et al.* have reported slightly lower drug contents for PLGA microspheres prepared from extraction process as compared to the evaporation process (35).

Some investigators have compared the microspheres produced from the o/o method against those produced from the o/w process (24, 39). Wada *et al.* have reported that o/o method gave L-PLA microspheres having smooth spherical surface and higher drug entrapment due to reduction in partitioning of drug in the external oil phase (24). The o/w process on the other hand gave a poor product with drug particles sticking out from the surface and poor drug entrapment (24). Contrary to these results, Menegatti *et al.* have stated that the o/w process produced microspheres having average size of 38.4 μm with no aggregation, as compared to the o/o method which yielded a poor product having severe aggregation (39).

The rate of temperature rise and the operating temperature for solvent evaporation strongly influences the microsphere product. Kyo *et al.* have reported that the solvent evaporation at the rate of 0.5 and 2.0° C/min in an o/o process yielded PLGA microspheres having many structural defects as against evaporation at 0.2° C/min which produced fewer defects (40). Wakiyama *et al.* have found that the organic solvent removal by heating at 40° C produced a viscous aqueous phase and resulted in relatively larger microcapsules, than those produced by removing solvent by vacuum at room temperature without any heat (69). Tice and Gilley have pointed out that very rapid DCM evaporation would cause DCM to boil off from the emulsion droplets, yielding microspheres with cracks and pin-holes (76). Jalil and Nixon have stated that when temperature of 85° C were used (above the boiling point of the solvent acetonitrile), highly

porous microspheres, having internal honey-comb like structure were produced (77). Van Hamont *et al.* found a predictable linear increase in the average PLGA microsphere size as the temperature of the continuous oil phase (various grades of machine oil) was increased from 20 to 30° C during the evaporation (of acetonitrile) phase of the o/o emulsification process (37). This linearity was lost as the temperature was increased from 30 to 40° C due to changes in the solubility of acetonitrile in oil (37). By heating an o/w emulsion for 2 hr at 50° C, Vaughan *et al.* could increase the drug loading efficiency from 20-30% to 75-85% (34). Evaluation of hydrocortisone stability in PLA microspheres at different temperature/time storage conditions revealed no drug degradation (25).

Generally, increasing the stirring rate decreases the microsphere size and narrows the size distribution (49). Crossan and Whateley prepared PLGA microspheres in the size range of 40-60 µm by using an overhead paddle stirrer and stirring for four hours at room temperature (53). Modification of this system by addition of a baffle reduced their size to 20-40 µm (53). A similar result was reported by Bodmeier and McGinity (78). The side baffles reduced the effective diameter of the vessel and hence lead to formation of smaller emulsion droplets. Also, the baffles reduced the turbulence in the suspension mixture, thereby increasing the stability of droplet suspension and the product yield. Further size reduction (5-10 µm) was achieved by first high speed stirring (1500 rpm) for 10 min using a Silverson homogenizer, followed by magnetic stirring for 18 hr. to enable complete evaporation of DCM (53). Rosilio *et al.* found out that for a drug loading of 0-30%, a stirring speed of 480 rpm was required and for a drug loading of 50-65%, stirring speed of 645 rpm was necessary to produce the microspheres (51). Coombes *et al.* have stated that, increasing the stirring rate of emulsion resulted in decrease in polydispersity of the PLGA microspheres but not in their particle size (44).

(2) Double (multiple) emulsion process

The double emulsion process is essentially an water-in-oil-in-water (w/o/w) method and is best suited to encapsulated water-soluble drugs like

peptides, proteins, and vaccines, unlike the o/w method which is ideal for water-insoluble drugs like steroids (1, 4, 5). A buffered or plain aqueous solution of the drug (sometimes containing a viscosity building and/or stabilizing protein like gelatin) is added to an organic phase consisting of PLGA and/or PLA solution in DCM with vigorous stirring to form the first microfine w/o emulsion. This emulsion is added gently with stirring into a large volume water containing an emulsifier like PVA to form the w/o/w emulsion. The emulsion is then subjected to solvent removal by either evaporation or extraction process. In the former case the emulsion is maintained at reduced pressure or at atmospheric pressure and stirred to enable DCM to evaporate. In the latter case the emulsion is transferred to a large quantity of water (with or without surfactant) with stirring, into which DCM is diffused out. The solid microspheres so obtained are then washed and collected by filtration, sieving, or centrifugation. These are then dried under appropriate conditions or are lyophilized to give the final free flowing microsphere product.

Some groups have reported using ethyl acetate as the polymer solvent and hydrophilic stabilizers like Pluronic F68, PEG 4600, BSA, HSA or sodium glutamate for protein/peptide drugs (79). Singh *et al.* used a blend of PVA and PVP in the outer aqueous phase to make PLA/PLGA microspheres (80). Cohen *et al.* have used an outer aqueous PVA phase saturated with DCM to prepare PLGA microspheres (81). Alpar *et al.* have reported preparation of PLA microspheres in which the inner aqueous phase contained MC besides PVA or PVP (82, 83). They found that particles containing PVP were more hydrophobic, exhibited higher drug loading and encapsulation efficiency, and showed decreased burst effect as compared to those containing PVA (82, 83). The addition of a stabilizing polymer (BSA), reduced the net encapsulation efficiency of the protein drug (82).

A number of hydrophilic drugs like the peptide leuprolide acetate, a luteinizing hormone-releasing hormone (LH-RH) agonist (84-89), vaccines (21, 79-81, 83, 90-124), proteins/peptides (82, 125-138), and conventional molecules

(139-151) have been successfully encapsulated by this method. Various formulation and process variables significantly affect the final microsphere product and the drug release from them.

(i) The primary w/o emulsion

Ogawa *et al.* have concluded that the encapsulation efficiency of the drug in PLA and PLGA microparticles increased with the increase in viscosity of the inner aqueous phase (containing gelatin) and also by increasing the viscosity of the whole w/o emulsion (by decreasing the amount of DCM) (84). The authors concluded that the high viscosity prevented the migration of the inner aqueous phase to the outer water phase due to local demulsification produced by the vigorous stirring (84). Similar results have been reported by Jeffery *et al.* who also found an increase in the microparticle size with the increase in viscosity of the inner aqueous phase (92). However increasing the viscosity of the inner aqueous phase by adding PVA had no effect on the drug entrapment or the particle size of the final microparticles (92). Jeffery *et al.* and others have reported that an increase in particle size and drug entrapment was observed following an increase in the internal aqueous phase volume (92, 118). In a study, Crotts and Park have stated that the volume of the inner aqueous phase drastically affected the morphology of the final microspheres and the subsequent drug release from them; those prepared from 5.6% aqueous phase fraction were dense and non-porous while those prepared from 22.7% aqueous phase fraction were porous in nature (118). Alonso *et al.* have reported that incorporation of a lipophilic surfactant, L- α -phosphatidylcholine (by dissolving it in chloroform and adding this solution to the DCM phase) produced more hydrophobic microspheres, causing reduction of the microsphere size and increase in particle porosity due to better stabilization of the inner w/o emulsion (100). Other researchers have also reported use of L- α -phosphatidylcholine (112). In another study it was found that a decrease in the DCM phase volume yielded particles with dense core (81).

The entrapment efficiency of the drug increased with decrease in drug loading and increase in particle size (84). However other groups have found no

relationship between encapsulation efficiency and drug loading (92, 104). Jeffery *et al.* have reported that an increase in the antigen/PLGA ratio resulted in increase in drug entrapment by PLGA and a small increase in the mean particle size of the final microparticles (92). Also an SEM analysis revealed that at low antigen/PLGA smooth particles were produced but at higher ratios the particles were pitted and some particles had collapsed (92). The authors attributed this to high surface concentration of antigen which became soluble in the surrounding external phase, leaving a pitted surface and in some cases this caused the microparticles to collapse (92). In another study small particles were produced when the volume ratio of DCM to PLA was low (84).

Jeffery *et al.* have reported the effect of hydrophobicity (molecular weight) of the polymer on the entrapment of the antigen; more hydrophobic (high molecular weight of 53K) PLGA, showed relatively lower entrapment levels of the drug than less hydrophobic 22K PLGA (92). However, Alonso *et al.* found no relationship in the encapsulation efficiency with respect to polymer composition (PLA v/s PLGA) and molecular weight (3K v/s 100K) (79). Okada *et al.* have reported that an increase in the content of water-soluble oligomers (free acid content) in PLA resulted in increase in the burst release of the encapsulated drug (87). Also increase in the Tg of the PLA and PLGA microspheres was observed with increase in drug loading (87). Alonso *et al.* have pointed out an increase in the microparticle size with increase in the molecular weight and the concentration of the polymer (79, 100). In a study, Benoit *et al.* found that microparticles prepared from PLGA were relatively larger and exhibited higher drug entrapment efficiency as compared to those prepared from PCL (124). Hilbert *et al.* used an aqueous liposomal suspension as the inner aqueous phase and prepared microencapsulated liposomes (109). These showed an higher burst effect as compared to the normal microspheres due to amphiphilic nature of the phospholipids which generated porous matrix surface (109). Sah *et al.* have reported that microcapsules containing PLA5000 (molecular weight 5000) or PLGA5000 (molecular weight 5000) into PLGA 75:25 microcapsules exhibited

increased degradation rates as compared to those containing PLGA 75:25 alone (117, 119, 120). The authors found that PLA5000 plasticized PLGA 75:25 and facilitated its faster degradation (120). Other groups have also reported the effect of PLGAs having different molecular weight and lactide/glycolide ratio on the final microparticle size, drug entrapment, and the degradation rate of the polymer (94, 106).

Microparticles loaded with greater amount of drug, gave a greater burst release of the drug due to increase in the number of channels formed by the hydrophilic drug (84, 87, 120). In a study, Alonso *et al.* have reported that the microparticles prepared by the double emulsion method (drug dissolved in the inner aqueous phase) produced more regular microspheres with better control over drug release, than those prepared by powder dispersion method (drug powder dispersed in DCM phase) (100). Reich has noted that the encapsulated protein drugs decrease the interfacial tension between the inner aqueous phase and the DCM phase of the o/w emulsion (123). The properties of the protein drug has a substantial effect on its entrapment and release thus leading to a different optimum for different protein/polymer combinations (123).

Cohen *et al.* have reported that for microspheres in which the inner emulsion was prepared using low shear (e.g. vortex mixing), the particles were large in size and the drug encapsulation was low as compared to microspheres in which the inner emulsion was prepared using high shear (e.g. probe sonication) which yielded smaller particles with higher encapsulation efficiency (81). However Sah *et al.* reported no effect of the shear rate (to prepare the o/w emulsion) on the encapsulation efficiency and the final particle size of PLA/PLGA microcapsules; particles prepared from low shear rate were however more porous than those prepared from high shear rate (116).

(ii) The double w/o/w emulsion

In a study Ogawa *et al.* have reported that smaller microparticles were produced when the mixing speed during emulsification of the w/o emulsion into the double w/o/w emulsion was increased (84). A similar result was reported by

Uchida and Goto, who also found a decrease in the drug loading efficiency with increase in the stirring rate (105). Also an increase in the external phase volume led to a decrease in the particle size of the microparticles (84). Jeffery *et al.* have reported an increase in the drug entrapment and the particle size with increase in the external aqueous phase volume (92).

Jeffery *et al.* found a reduction in particle size as the concentration of PVA increased in the external aqueous phase; the entrapment of the drug was however not affected (92). The authors attributed this to unstable emulsion droplets at low PVA concentration resulting in formation of larger microparticles as compared to those prepared from high PVA concentration (92). Singh *et al.* investigated the residual PVA content in PLGA microparticles and concluded that various process parameters like volume and concentration of the aqueous PVA solution and the number of washes in the microencapsulation process could control the residual levels of PVA within the acceptable limits (99).

Alonso *et al.* have compared microsphere preparation by two methods in which the final organic solvent (DCM) was removed by evaporation and by extraction into 2% aqueous isopropanol solution; no major difference was found in the physical characteristics and the controlled drug release properties of the resultant microspheres. The extraction technique however, yielded the microspheres in only 30 min (79, 100). Other groups have also reported use of 2% aqueous isopropanol solution to remove the solvent (113).

(iii) Drugs

Researchers at Takeda Chemical Industries have reported successful encapsulation of leuprolide acetate, a luteinizing hormone-releasing hormone (LH-RH) agonist (for treating endometriosis) into PLGA microparticles by the double emulsion method (84-89). A pseudo-zero order release profile (for 1 month) after administering PLGA loaded leuprolide acetate in rats through s.c. and i.m. routes (85, 86, 88) and a three-month release profile following a s.c. injection (87) has been reported by these researchers.

There is a lot of interest in delivering vaccines through PLA/PLGA microparticles and immense literature has been published on this aspect (13, 16, 28, 30, 32-36, 38, 40, 41, 54, 55, 64, 65, 74, 100, 102, 106-109, 114, 142, 155). A number of vaccines/immunogenic agents like ovalbumin (30, 32-36, 42, 64, 65, 155, 160), *Dermatophagoides Pteronysinus* for hyposensitization therapy (38, 40), cholera toxin (41), tetanus toxoid (79, 100, 106, 114), ricin toxoid (102), HIV vaccine (102), birth control vaccine (107, 109), BSA (39, 54, 55, 81, 95, 124, 139, 140-143, 149, 151, 160), influenza toxin (28), rotovirus (13), adenovirus (16), lysozyme (39), and *Schistosoma mansoni* against Schistosomiasis (93) have been successfully delivered by encapsulation into PLA/PLGA microparticles. These have been delivered by s.c., i.m., and oral route to provide pulse as well as sustained immune response for days, weeks, and months.

Besides vaccines, other peptide/protein based drugs and certain synthetic drugs have also been successfully loaded into PLA/PLGA microparticles by the double emulsion method and administered for prolonged release effect (82, 125-151).

B. Phase Separation (Coacervation)

The coacervation method consists of decreasing the solubility of the encapsulating polymer by addition of a third component to the polymer solution in an organic solution (1, 4, 5). At a particular point, the process yields two liquid phases (phase separation): the polymer containing coacervate phase and the supernatant phase depleted in polymer. The drug which is dispersed/dissolved in the polymer solution is coated by the coacervate. Thus the coacervation process includes the following three steps: (i) phase separation of the coating polymer solution, (ii) adsorption of the coacervate around the drug particles, and (iii) solidification of the microspheres (152).

First, the polymer is dissolved in an organic solution. The water-soluble drugs like peptides and proteins are dissolved in water and dispersed in the polymer solution (w/o emulsion). Hydrophobic drugs like steroids are either solubilized or dispersed in the polymer solution. An organic nonsolvent is then

added to the polymer-drug-solvent system with stirring which gradually extracts the polymer solvent. As a result the polymer is subjected to phase separation and it forms very soft coacervate droplets (size controlled by stirring) which entrap the drug. This system is then transferred to a large quantity of another organic nonsolvent to harden the microdroplets and form the final microspheres which are collected by washing, sieving, filtration, or centrifugation, and are finally dried (4, 152).

The phase separation method, unlike the o/w emulsification method is suitable to encapsulate both water-soluble as well as water-insoluble drugs, since it is a non-aqueous method. However the coacervation process is mainly used to encapsulate water soluble drugs like peptides, proteins, and vaccines. The addition rate of first nonsolvent should be such that the polymer solvent is extracted slowly, so that the polymer has sufficient time to deposit and coat evenly on the drug particle surface during the coacervation process (4). The concentration of the polymer used is important as well, since too higher concentrations would result in rapid phase separation and nonuniform coating of the polymer on the drug particles. Due to absence of any emulsion stabilizer in the coacervation process, agglomeration is a frequent problem in this method (4). The coacervate droplets are extremely sticky and adhere to each other before the complete phase separation or the hardening stages of this method. Adjusting the stirring rate, temperature, or the addition of an additive is known to rectify this problem (4).

Unlike the solvent evaporation/extraction process, the requirement of solvents for the polymer are less stringent since the solvent need not be immiscible with water and the boiling point can be higher than that of water (4). DCM, acetonitrile, ethyl acetate, and toluene have been used in this process (152-163). The nonsolvents affect both the phase separation and the hardening stages of the coacervation process. The nonsolvents should not dissolve the polymer or the drug and should be miscible with the polymer solvent (152-160). The second nonsolvent should be relatively volatile and should easily remove the first viscous

nonsolvent by washing. Some of the oils used as the first nonsolvent are silicone oil, vegetable oils, light liquid paraffin, low molecular weight liquid polybutadiene, and low molecular weight liquid methacrylic polymers (4, 152-163). Examples of the second nonsolvent include aliphatic hydrocarbons like hexane, heptane, and petroleum ether (4, 152-163).

In the coacervation process the phase equilibrium is never reached and hence the system is constantly out of equilibrium (4). Therefore the formulation and process variables significantly affect the kinetics of the entire process and ultimately the characteristics of the final microspheres. In a classic article, Nihant *et al.* have investigated the effect of several process factors on the coacervation process (152). With increase in the aqueous phase/organic phase volume ratio from 0.02 to 0.12% w/w the 'stability window' (an area in the phase diagram where the dispersed aqueous phase is efficiently coated by the coacervate) was unmodified and only got slightly narrower (152). An SEM picture revealed that the morphology of the particles changed from a spherical shape for 0.02 ratio to a deformed one at higher ratio of 0.12. Above water contents of 0.12, the microspheres became brittle and spontaneously released the encapsulated drug solution during filtration (152). With decrease in the stirring rate from 800 to 400 rpm for the aqueous drug dispersion in PLGA/DCM solution, the particle size increased from 40.0 to 51.5 μm and for 300 rpm no microparticles were formed (152). Similarly with decrease in the stirring rate from 200 to 130 rpm for the phase separation by adding silicone oil, the particle size increased from 40.0 to 58.0 μm and for 100 rpm no microparticles were formed. For the addition rate (of silicone oil) of 18 ml/min microparticles of the size 40.0 μm were formed and their size decreased to 39.1 μm when the addition rate was decreased to 5.7 ml/min (152). However, with further decrease in the addition rate to 0.65 ml/min, the particle size increased to 53.1 μm and aggregates were formed and in certain cases no microparticles were formed. The authors concluded that microencapsulation by coacervation is a complex process that depends on the interplay of several kinetic parameters (152). In another paper, the same group

has reported the effects of weight, volume, composition, and viscosity of the coacervate and supernatant phases on the size distribution, surface morphology, and internal porosity of the final microparticles (162).

Other groups have also reported microencapsulation by coacervation (153-161, 163). Vidmar *et al.* induced phase separation of a drug-PLA-DCM suspension by addition of n-heptane to give particles in the range of 50-500 μm and in another study they used chloroform instead of DCM to dissolve the polymer (1). Nakano *et al.* used an ethyl acetate solution of PLA/carboxymethyl ethyl cellulose blend and suspended the drug particles in it prior to inducing phase separation by adding ethyl ether to finally give smooth microspheres having mean size of 16.4 μm (1). Fong *et al.* carried out microencapsulation at low temperature, where the drug was suspended in PLA/toluene solution at -65°C and phase separation was induced by dropwise addition of isopropanol with constant stirring to yield microspheres in the range of 25-50 μm (164). Mandal *et al.* added the suspension of water soluble diltiazem or metoprolol in PLGA/DCM solution to a silicone oil:DCM solution (1:6 ratio) with stirring and the coacervates obtained were hardened by petroleum ether to yield microspheres with high encapsulation efficiencies (153). In an article, Ruiz *et al.* have concluded that the polymer properties such as hydrophobicity or chain length, viscosity of the silicone oil used, the concentration of the polymer, and the polymer solvent/silicone oil ratio greatly affected the overall coacervation process and thereby the characteristics of the final microsphere product (165). Leelarasamee *et al.* have reported preparation of PLA microcapsules by solvent partitioning to achieve phase separation (159). A solution of hydrocortisone and PLA in DCM was slowly injected into a mineral oil stream with a constant injection rate and needle size. As DCM partitioned into the mineral oil phase, the polymer precipitated and encapsulated the drug. The microcapsules were finally washed with hexane and they had a size of 250 μm with 90% yield (159).

C. Spray Drying

Injectable biodegradable PLA and PLGA microparticles have been successfully prepared by double emulsion and phase separation as discussed in the previous sections. The coacervation method tends to produce particles which are agglomerated, there is difficulty in mass production, the method requires large quantities of organic solvent, and it is difficult to remove residual solvents from the final microsphere product (166). The double emulsion method, on the other hand, requires many steps, rigid control of the temperature and viscosity of the inner w/o emulsion, and is difficult to encapsulate higher concentration of hydrophilic drugs (1, 166). Contrary to these methods, the spray drying method is very rapid, convenient, easy to scale-up, involves mild conditions, and is less dependent on the solubility parameter of the drug and the polymer (1, 166, 167).

Wise *et al.* reported the preparation of PLGA microcapsules in which, a solution of PLGA, hexafluoro-2-propanol, benzene, and the drug was sprayed to produce particles of less than 125 μ m (168). Bodmeier and Chen prepared microspheres by spray drying where a water soluble drug (theophylline) was suspended or a water-insoluble drug (progesterone) was dissolved in a PLA/DCM solution and then spray dried to produce particles of less than 5 μ m (169). Due to incompatibility of the hydrophilic drug and PLA, needle shaped crystals grew on the microsphere surface, while the progesterone-PLA solution gave smooth particles. The nature of the solvent used, temperature of the solvent evaporation, and presence of PLA microspheres during the spray drying process affected the polymorphic form of progesterone. A major problem encountered with this technique was the formation of fibers due to insufficient force available to breakup the polymer solution. An efficient dispersion of the filament into polymer droplets was dependent on the type of polymer and the viscosity of the spray solution. Other groups have also reported successful preparation of PLGA and PLA particles using the spray drying technique (167, 170-174).

Wagenaar and Müller spray dried a solution of the polymer, DCM, and the drug piroxicam to yield microspheres which were hollow (no solid core) (167). DL-PLA microparticles were more spherical and smooth than those made from

DL-PLGA. The microspheres were in the size range of 1 to 15 μm , with an high drug encapsulation efficiency of 99.0% (167). Men *et al.* have shown that PLGA microparticles prepared using spray drying technique produced particles in the size range of 1 to 15 μm and drug loading of 4.4 to 6.6 $\mu\text{g}/\text{mg}$ microspheres, while PLA microspheres prepared using coacervation technique yielded particles having diameter in the range of 20 to 90 μm with relatively low drug loading of 3.5 $\mu\text{g}/\text{mg}$ microspheres (173). In order to protect an hepatitis vaccine from the harmful effects of the solvent, a mixture of the antigen powder and an hydrophilic polymer, Hydroxypropyl cellulose (HPC) was first spray dried to produce core microparticles (174). These were then suspended in PLGA/ethyl acetate solution and spray dried to yield double walled microparticles in the size range of 4 to 22 μm . The first coating layer of HPC protected the antigen from solvent during the second encapsulation process with PLGA (174).

Spray drying method might cause a significant loss of the product due to adhesion of the microparticles to the inside wall of the spray drier apparatus, and can also produce agglomeration of the microparticles (166). In order to rectify these problems, a novel double-nozzle spray drying technique was developed which involved use of mannitol as an anti-adherent (166). A solution or a dispersion (w/o emulsion) of the drug in PLGA solution was sprayed from one nozzle and from another nozzle an aqueous mannitol solution was sprayed simultaneously and the process completed to give the final microspheres. The surface of the spray dried microspheres were coated with mannitol and the extent of agglomeration was decreased (166). Also this method produced microspheres with higher yield and encapsulation ratio as compared to those prepared from the double emulsion method (166).

D. Miscellaneous Methods

Drug-free microsphere preparation by an interfacial deposition technique has been described by Makino *et al.* (175). First, 50 ml of n-hexane was emulsified in 1.5% w/w aqueous Pluronic F68 solution to yield an o/w emulsion. Then 20 ml of 1.5% w/v solution of D,L-PLA or L-PLA in DCM was added

dropwise to the emulsion with stirring, resulting in precipitation of the polymer and formation of 1.5 μm size microcapsules on complete solvent evaporation (175). Wichert and Rohdewald have prepared microparticles by a new melting method (176). The polymer D,L-PLA (molecular weight 2000 or 16,000) was first melted in presence of the drug vinpocetine at 180° C, to obtain a homogeneous mixture. This was then emulsified in a hot aqueous Tween 80 solution. Microspheres were obtained from this emulsion by either centrifugation or spray drying. For the first method, the emulsion was poured into an ice water and the suspension subsequently centrifuged and the particles obtained were then lyophilized. For the second method, PVA was added to the hot emulsion and the emulsion then spray dried to obtain the final microparticles. The particle size depended on the molecular weight of the polymer and the isolation method and were found to be in the range of 2-22 μm . The authors also found that during the microparticle preparation, the molecular weight of PLA reduced by 12% (176).

A novel low temperature method for preparing PLA and PLGA microspheres has been reported by Khan *et al.* (177) and the group at Alkermes, Inc. (178, 179). First the protein powder and optional excipients were suspended in the PLA/PLGA solution in acetone, ethyl acetate, or DCM. This suspension was then sprayed into a vessel containing liquid nitrogen overlaying a frozen extraction solvent like ethanol. The liquid nitrogen evaporates, allowing the polymer solvent from the frozen droplets to be extracted by, liquid ethanol, now turned liquid. Microspheres were then filtered and the residual solvents evaporated. The microspheres were 50-60 μm in size with drug encapsulation efficiency more than 95% (177-179). Sam *et al.* have described a new spray desolvation method using non-toxic solvents (180). The micronized drug was first suspend in a PLGA solution in acetone. This suspension was then atomized ultrasonically in an ethanol bath to induce coagulation of droplets. After 30 minutes, ethanol was replaced by water to cause hardening of PLGA, and the microparticles so formed were then collected and dried under vacuum. The volume average diameter of the microparticles was 77 μm with drug

encapsulation efficiency of 30-60% for water-soluble hexapeptides and 90% for water-insoluble estradiol (180).

Iwata and McGinity have reported preparation of multi-phase microspheres of D,L-PLA and D,L-PLGA containing water-soluble drugs (181, 182). An aqueous drug solution containing gelatin and Tween 80 was emulsified into soybean oil containing aluminum monostearate and Tween 80 (o/w emulsion). This was dispersed in PLA/PLGA solution of acetonitrile to form the double w/o/"w" emulsion. This was then dispersed in light mineral oil containing Span 80 to form the w/o/"w"/o multiple emulsion which was then agitated to evaporate and remove acetonitrile. The hardened microspheres were filtered and washed with n-hexane and water and finally dried under reduced pressure. Encapsulation efficiencies of 80-100% were obtained by this method and were higher as compared to microspheres made by the conventional o/o-solvent evaporation method, where the drug partitioned in the polymer-acetonitrile phase (181, 182).

To delay the release of protein from PLGA microspheres and to maintain its biological activity, Madiba and Wu *et al.* combined the PLGA microspheres with BSA loaded liposomes (183). The authors first prepared protein loaded liposomes consisting of 1:2 molar ratio of cholesterol and dioleoylphosphatidylcholine. Then a 6% w/w PLGA (75:25) solution in ethyl acetate was made. The above liposome suspension was added to the polymer solution and the mixture homogenized to produce a w/o emulsion. This emulsion was then transferred to 85 ml of 3% w/v aqueous PVA solution, containing 5 ml ethyl acetate and the mixture stirred to form the double w/o/w emulsion. The solvent was removed by extracting twice in an ice-cold water and the microspheres so obtained were finally filtered and dried and were found to be in the range of 30-100 μm . The authors did not provide any information on the drug encapsulation efficiency or the stability of the encapsulated BSA (183).

To prevent water-mediated inactivation of encapsulated vaccines, an oil-based PLGA microcapsules were designed, consisting of an oily core of antigen

(tetanus toxoid), in which the protein is dispersed surrounded by an outer PLGA shell (184). The antigen powder was first dispersed in mineral oil (1:100 powder:oil ratio) and the microcapsules then obtained by the conventional o/w-solvent extraction/evaporation method. Microcapsules prepared using 50:50 PLGA had a mean size of 112 μm and an encapsulation efficiency of 63%, while those made from 75:25 PLGA were 129 μm in mean size with an encapsulation efficiency of 86% (184). Hariharan and Price have reported encapsulation of the protein, OVA by a novel colloidal suspension method (185). First OVA was precipitated out of water using acetone to produce a colloidal suspension of OVA. The water was then removed by dialysis and PLGA (50:50) was dissolved in the acetone suspension, which was then emulsified in heavy mineral oil in presence of magnesium stearate. Evaporation of solvent produced microspheres containing colloiddally dispersed OVA. The microspheres were 90-180 μm in size and had encapsulation efficiency of 89-94% (185). Abraham and Burgess have described PLGA microsphere preparation using a novel rapid solvent evaporation method (186). The drug, chlordiazepoxide and PLGA were first dissolved in DCM and then dispersed in aqueous 0.01% PVA solution to give an o/w emulsion. Water at 70° C was added to this mixture, thereby raising the temperature of the system above the boiling point of DCM, causing its rapid evaporation and subsequent formation of PLGA microspheres (186).

Burns *et al.* have described a new continuous encapsulation process for preparation of progesterone and estradiol containing D,L-PLA microspheres (187). The process was the same as the traditional o/w-solvent evaporation method, except that it had been converted from a batch mode to a more cost efficient continuous process, which according to the authors, reduced several problems associated with scale-up and process validation. There have also been reports of preparation of PLA and PLGA microparticles by using supercritical fluid (compressed carbon dioxide) extraction/expansion techniques (188).

III] NANOPARTICLES

Injectable microparticles from PLA and PLGA have been successfully prepared to deliver drugs like peptides, proteins, and vaccines over a period of days, weeks, or even months at a constant rate depending upon the degradation behavior of the polymer employed. However due to their large size, it was impossible to direct the drug to target tissues via systemic circulation or across the mucosal membrane (11). Following oral administration, particles less than 500 nm can cross the M cells in the Payer's patch and the mesentery on the surface of the gastrointestinal tract mucosa, delivering the drug to the systemic circulation (11).

Nanospheres and nanocapsules could be prepared by the same methods as those described for microparticles, except that manufacturing parameters are adjusted to obtain nanometer size droplets (10). This is obtained by using a relatively small ratio of the dispersed phase to the dispersion medium and a substantially higher stirring speed (10). Song *et al.* have prepared PLGA nanoparticles by o/w emulsification/solvent evaporation technique to produce nanoparticles having size of 150 nm, drug loading of 15.5% w/w, encapsulation efficiency of 62% and yield of 85% (190). Many formulation/process variables affecting microsphere production also influence nanoparticle production, in more or less the similar way. Dawson and Halbert prepared nanoparticles by the o/w emulsification technique and used response surface methodology to determine the effect of some variables on the size distribution of PLGA nanoparticles (191). The authors found that the homogenization pressure and PLGA concentration have a linear and thus predictable effect on both size and polydispersity of the particles. Tween 80 concentration and the DCM (organic phase) concentration had a greater effect on the diameter and polydispersity (191).

Müller *et al.* have described a novel method of preparing PLA and PLGA nanoparticles (192). Magnetite (used for magnetic resonance imaging) was first dispersed in ethanol by sonication. This dispersion was then incorporated in the polyester polymers and the temperature slowly increased with stirring to remove ethanol (192). Heating and stirring was continued to melt PLA/PLGA polymer to

facilitate homogeneous dispersion of magnetite in them. After solidification of the magnetite-containing polymer, they were grounded (192). PLA/PLGA nanoparticles were then obtained by high pressure homogenization of the magnetite-containing polymer particles dispersed in aqueous poloxamer 188 solution (192). Allemann *et al.* prepared PLA nanoparticles by a reversible salting-out process using a cross-flow filtration technique which involved the use of magnesium salts (193). A 90% drug entrapment was achieved by this method.

Niwa *et al.* have reported nanospheres production for indomethacin and 5-fluorouracil (water- insoluble and water soluble drugs respectively) and for nafarelin acetate (NA), a LH-RH analog by a novel spontaneous emulsification-solvent diffusion method (194, 195). The drug and PLGA were first dissolved in acetone-DCM mixture and then emulsified in an aqueous PVA solution using a high-speed homogenizer (o/w emulsification). The rapid diffusion of acetone in the aqueous phase resulted in faster deposition of polymeric film on the droplet and yielded nanospheres less than 500 nm (194, 195). The same group also reported nanospheres production for NA by the emulsion-phase separation method in an oil system (196). An aqueous solution of NA was emulsified in acetone-DCM mixture containing dissolved PLGA using a homogenizer at 15,000 rpm. To this was added a mixture of Triester oil (caprylate and caprate triglyceride) and HGCR (hexaglycerine condensed ricinoleate), which resulted in phase separation of PLGA. PLGA coacervates precipitated around the aqueous emulsion droplets and were hardened by evaporation of the solvent to yield nanospheres in the range of 500-800 nm (196). Murakami *et al.* have reported preparation of PLGA latex by a method based on double coacervation of PVA and PLGA (197). PLGA was first dissolved in a mixture of acetone and DCM, ethanol, or methanol. This PLGA solution was then dispersed in aqueous PVA solution with stirring (197). The latex dispersion was then freeze dried to obtain powder latex having size of approximately 300 nm. The authors concluded that the coacervated (adsorbed) PVA molecules prevented the aggregation of PLGA nanoparticles due to its steric hindrance (197).

Following intravenous administration, the nanoparticles are taken up by cells of the mononuclear phagocyte system mainly the Kupffer cells of the liver and the spleen macrophages and are essentially lost (198, 199). This is considered as the major hurdle to target delivery of drugs to other organ/tissue sites within the body (198, 199). Several groups have tried to address this problem. Gref *et al.* prepared polyethylene glycol (PEG)-coated nanospheres by employing an amphiphilic diblock copolymer of PLGA (75:25, lactide:glycolide) and PEG (molecular weight 5000-20,000) (198). The nanospheres were prepared by a single-step o/w solvent evaporation technique, where the PEG fraction migrated to the surface of the nanospheres forming a protective cover. After 5 minutes of the injection of coated or uncoated nanospheres, 15% of coated nanospheres were found in the liver and 60% of them in the blood with a significantly improved circulating time (198). In comparison, 40% of uncoated nanospheres were found in liver and only 15% of them in the blood. Also 4 hours post-injection, 30% of coated nanospheres were still circulating in the blood while the plain nanospheres had completely disappeared from the circulation. Blood circulation time for the nanoparticles increased as the molecular weight of the PEG increased from 5000 to 20,000 due to increased thickness of the protective PEG layer which prevented their opsonization (198). Stolnik *et al.* (199) and Dunn *et al.* (200) reported preparation of PLGA nanospheres by o/w-emulsification/solvent evaporation and further coating them by the diblock copolymer PLA:PEG (ratio of 2:5 and 3:4). The coated particles had increased hydrophilicity and decreased surface charge (as determined by measuring their surface zeta potentials) and were sterically stabilized particles. These particles exhibited reduced protein adsorption and liver uptake and increased blood circulation time as compared to uncoated PLGA nanospheres (199, 200). Leroux *et al.* have also reported similar improved site specific drug delivery of nanoparticles after coating them with PEG (193). Other groups have also reported successful preparation of PLGA nanoparticles (201-204).

[III] OTHER DEVICES

Many groups have reported preparation of drug-loaded implants from PLA or PLGA (205-209). Kunou *et al.* have designed nail-like ganciclovir (GCV) incorporated D,L-PLGA implant (length-5mm, diameter-1mm) for intraocular drug delivery to treat cytomegalovirus retinitis (210). Wang *et al.* have reported preparation (by compression) of 5-fluorouracil D,L-PLGA subconjunctival coated and uncoated implants/matrices (5 mg, diameter-2.5mm, thickness-1.2mm) using drug:PLGA ratio of 9:1, 8:2, and 7:3 (211).

In order to study the release profile of zidovudine (AZT), Mandal *et al.* prepared tablets by compressing physical mixture of AZT and PLGA (50:50) and AZT loaded PLGA microcapsules (212). Other groups have also reported preparation of PLA/PLGA compressed tablets with some involving use of heat (213-215).

Schmitt *et al.* have reported preparation of amaranth incorporated pellets (diameter-3.7 mm, thickness-3.1 mm) from D,L-PLGA which was purchased from three different sources: DuPont, Birmingham Polymers, and Henley Chemicals, Inc. (216). The authors prepared the pellets by melt-pressing spray-dried PLGA with a 4-mm standard concave punch and die set (216). Tracy *et al.* prepared drug-free PLGA pellets by a similar method but used a Carver Laboratory Press instead (217).

In an article Schade *et al.* have described preparation of aqueous colloidal D,L-PLGA dispersion by a spontaneous emulsification-solvent diffusion technique followed by drying of these dispersions to form biodegradable latex films (218). In order to study the effect of hydrophilic excipient on the drug release from the hydrophobic PLGA (50:50) film, Song *et al.* prepared double-layer films (150 μm thickness), where the drug releasing layer consisted of drug/hydrophilic additive/PLGA (10:10:80 ratio) and a protecting layer consisting of PLGA only (219). A combined solvent casting and melt-compression method was utilized to prepare these films (219). In an effort to study drug-induced D,L-PLA hydrolytic degradation, Li *et al.* prepared caffeine incorporated PLA circular

plates (1.5 mm thick) and films (0.3 mm thin) (220). Other researchers have also described drug delivery through PLGA films (221, 222).

Hsu *et al.* have reported preparation of low density PLGA (85:15) foams, having high interstitial void volume. First PLGA was dissolved in glacial acetic acid at different concentrations (223, 224). The solutions were then frozen and lyophilized and the solvent recovered in a dry ice/acetone cooled trap to give foams which had leaflet or platelet structures. Lyophilization helped to achieve control of the specific gravity and interstitial void volume of the foam. The drug isoniazid (INH) was then impregnated into the foams, by immersing a weighed quantity of foam into an aqueous INH solution of known concentration and finally lyophilizing them to remove water. The foams were then subjected to matrices preparation by different methods using high pressure extrusion to prolong *in vitro* release of INH and to understand the mechanism of its release (223, 224). Lee *et al.* have described preparation of biodegradable drug incorporated L-PLA porous membranes (10 mg, 1 cm x 1 cm) for periodontal therapy by an in-air drying phase inversion technique which involved use of solvents like DCM and ethyl acetate (225). Whang and Healy have described preparation of BSA incorporated D, L-PLGA scaffolds having different pore sizes and very high porosities by an emulsion freeze-drying method (226). Pore size was controlled by varying polymer inherent viscosity and/or volume fraction of the dispersed aqueous phase and/or polymer concentration (w/v %) (226). Ovalbumin loaded D,L-PLA granules were prepared by first emulsification (o/w emulsion) of the vaccine followed by lyophilization of the emulsion and then compression-molding the powder into rods (227). These were then grounded by pestle and mortar into granules which were then sized by using appropriate sieves (20 to 100 μm) (227, 228).

[IV] IN SITU FORMED IMPLANTS

The manufacturing processes for PLGA microparticles discussed in the previous sections suffer from drawbacks such as: (i) the microspheres need to be reconstituted (suspended) in an aqueous media, before they could be injected in

the body, (ii) the hazards and environmental concern associated with the use of organic solvents like methylene chloride for the solubilization of PLGA polymer, and (iii) residual organic solvents remaining in the final microsphere product. Although PLGA implants have been fabricated to deliver a variety of drug classes, they have not received much commercial success, primarily due to difficulty in administration; they require minor surgical incision or special type of pellet injector (trocar), thereby causing inconvenience to the patients.

In order to improve patient acceptance a novel implant system has been developed which is intramuscularly or subcutaneously administered as a liquid and subsequently solidifies *in situ* (229, 230). First PLA or PLGA is dissolved by heating in a water miscible, biocompatible solvent (this may also act as a plasticizer for the polymer). The polymer solution is then cooled under ambient condition and the drug is dispersed into it by homogenization or alternatively, the drug is dissolved in a solvent (like propylene glycol) which is miscible with the polymer solvent and water. This polymer-solvent-drug system has a viscous consistency but is sufficiently syringeable to be enabled to be injected intramuscularly or subcutaneously by conventional syringe and needle. When injected, it comes in contact with water from aqueous buffer (*in vitro* condition) or physiological fluid (*in vivo* condition) and as a result the polymer precipitates and forms a gel matrix (solidifies) entrapping the drug (*in situ/in vitro* or *in situ/in vivo* implant formation). The polymer solvent dissipates and diffuses out of the system and water diffuses into the polymer matrix. Due to water insoluble nature of the polymer, it precipitates/coagulates to form a solid implant *in situ*, from which the drug is released in a controlled fashion.

A number of groups including one at Atrix Laboratories have extensively reported drug delivery using this method (229-246). These researchers have employed combination of a host of biocompatible solvents and biodegradable polyesters besides PLA and PLGA to deliver a variety of therapeutic drug classes (229-246).

Although this implant system precludes the need for any surgery for its administration, it has a number of disadvantages: (i) the safety of solvents like NMP used to formulate these systems is questionable and not well documented, (ii) the injection of these liquid implant systems and their subsequent solidification produce non-uniform matrix implants having variable consistency and geometry, and (iii) due to formation of matrix implants having inconsistent texture, shape and size, the drug release from them is variable and unpredictable. An alternative and attractive approach therefore would be to use non-aqueous solvents to produce *in situ* microspheres.

STERILIZATION OF PLGA BASED DEVICES

Sterilization of the final formulation containing the lactide and/or glycolide polymers is an important issue often overlooked in the early stages of drug delivery system development. Terminal sterilization and aseptic processing are two main methods reported for sterilization of PLGA based products.

Steam sterilization usually involves subjecting the product to steam at 121° C for at least 20 min or 115° C for 30 min (5, 12). This method cannot be used with PLGA systems because at higher temperature/pressure condition the polymer softens, melts and leads to deformation of the matrix form, and undergoes hydrolysis (4). Heat sterilization involves exposing the product to higher temperatures for longer periods of time which are destructive to both the polymer and the entrapped drug (12). Sterilization using a gas like ethylene oxide can be achieved when heat/steam sterilization are harmful to the formulation (4, 5, 12). However ethylene oxide has known to soften and plasticize these polymers. Also the residual gas vapors left in these device were found to be mutagenic, carcinogenic, and allergic (4, 5).

Radiation sterilization (^{60}Co γ rays) has been used in several cases to sterilize formulations containing lactide and/or glycolide polymers (4, 5, 12). Effect of radiation on PLGA has been a subject of various investigations (26, 53, 57, 59). Subjection of PLGA to γ irradiation produced dose dependent polymer

chain break down, molecular weight loss (decrease in inherent viscosity), increased *in vitro* and *in vivo* bioerosion rates, and increased drug release kinetics (59). Crossan and Whateley (53) and Spenlehauer *et al.* (247) have reported that irradiation by γ -rays did not in any way influence the drug release rates. In another study, exposure of PLA to 100 Gy γ -rays using ^{60}Co did not affect the microsphere structure, release rate, or the drug stability (26).

Aseptic processing is an effective but somewhat expensive technique for formulations containing PLGA polymers (4, 5, 12). Due to excellent solubility of these polymers in a number of organic solvents, they can be filter-sterilized. The drug delivery system can then be formulated in a clean room environment using Good Manufacturing Practice (GMP) protocols.

SUMMARY

Drug delivery from biodegradable PLGA polymers have generated immense interest due to their excellent biocompatibility and biodegradability. Also they are easy to formulate into drug carrying devices and have been approved by the FDA for drug delivery use. The various biodegradable PLGA devices fabricated from different techniques are versatile in terms of the various classes of drugs encapsulated, the different time period of their release, and the diverse routes of their delivery. PLGA microparticles, in particular, are important drug delivery systems where various drug release profiles can be achieved by adjusting the PLGA composition, molecular weight, drug loading, microparticle size, porosity, and other factors. The newer techniques for fabricating PLGA devices like the *in situ* formed implants are evidence of continued efforts by researchers throughout the world to optimize the drug delivery through PLGA polymer. Some drugs formulated into PLGA microparticles and other devices have already been introduced into the market and many more are undergoing clinical trials.

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MANUSCRIPT II

CONTROLLED DELIVERY OF DRUGS FROM A NOVEL INJECTABLE *IN*
SITU FORMED BIODEGRADABLE PLGA MICROSPHERE SYSTEM

ABSTRACT

A novel method for *in situ* preparation of injectable biodegradable microspheres from the copolymer poly(lactide-*co*-glycolide) (PLGA) without incorporating unacceptable organic solvents is described. The delivery system is a dispersion of PLGA microglobules ("premicrospheres" or "embryonic microspheres") in an acceptable vehicle mixture (continuous phase) and whose integrity is maintained by use of appropriate stabilizers. A solution of PLGA, triacetin, a model protein (cytochrome *c*), PEG 400, and Tween 80 (Oil Phase 1) are added dropwise with continuous homogenization to miglyol 812-Span 80 solution (Oil Phase 2), thereby inducing phase separation (coacervation) of PLGA and forming PLGA microglobules (containing cytochrome *c*) dispersed in the continuous phase. This novel drug delivery system (NDDS) is a dispersion and has a viscous consistency, but is sufficiently syringeable. When injected, it comes in contact with water from aqueous buffer or physiological fluid and as a result, the microglobules harden to form solid matrix type microparticles entrapping cytochrome *c* (*in situ* formed microspheres). Cytochrome *c* is then released from these microspheres in a controlled fashion. The composition, rationale, and optimization of the NDDS is described here. Various formulation variables such as the PLGA concentration and type and the substitution of the continuous phase by a fresh Oil Phase 2 influenced the characteristics of this system. The characteristics of the NDDS were reproducible and were not affected by a 15 days/4° C storage condition. Also, the formulation, process, and storage (15 days/4° C) conditions did not adversely affect the physical stability of the encapsulated cytochrome *c*.

INTRODUCTION

The biodegradable thermoplastic aliphatic polyesters like poly(lactide) (PLA), poly(glycolide) (PGA), and especially the copolymer of lactide and glycolide referred to as poly(lactide-*co*-glycolide) (PLGA) have generated immense interest due to their excellent biocompatibility and biodegradability [1-17]. Also PLGA has been approved by the FDA for a number of clinical applications including surgical sutures and as controlled-release microspheres [3, 4]. PLGA is shown to be biocompatible and degrades to toxicologically acceptable lactic and glycolic acids that are eventually eliminated from the body [1].

There is a particular interest in controlled delivery of macromolecules such as peptides and proteins through PLGA microspheres [5]. Although a wide variety of pharmacologically useful peptide and protein based drugs have been recently developed by genetic engineering, their therapeutic use is restricted due to certain disadvantages: [4, 5]: (1) on oral consumption they are subject to attack by the acidic and enzymatic environment in the stomach and the enzymes from the brush border membrane of the intestine, (2) their high molecular weight and size impede their effective transport across the gastrointestinal membranes, and (3) they have a short biological half-life and on injection they are quickly metabolized and eliminated. To achieve sustained blood levels of these drugs, prevent their denaturation or degradation, and to extend their biological half-life, their delivery by encapsulation in PLGA microspheres has become an interesting approach [4, 5].

The literature on PLGA microspheres is full of different techniques describing their manufacture, where the microspheres are produced in a free-flowing, powder form. Some of the methods reported are [1, 2, 4, 5]: (1) single/double emulsification followed by solvent removal by evaporation or extraction, (2) phase separation (coacervation), and (3) spray-drying. Most of these manufacturing processes suffer from drawbacks such as [1, 2, 4, 5]: (1) the microspheres need to be reconstituted (suspended) in an aqueous media before

their injection in the body, (2) the hazards and environmental concern associated with the use of organic solvents like methylene chloride (dichloromethane) (DCM) for the solubilization of PLGA polymer, and (3) residual organic solvents remaining in the final microsphere product.

Shah *et al.* [18] and researchers from Atrix Laboratories, Inc. (Fort Collins, CO) [19-26] have described a novel implant system which is parenterally administered as a liquid and subsequently solidifies into a gel matrix (implant) *in situ*, from which the drug is released in a controlled manner. Although this implant system precludes the need for any surgery for its administration, it has a number of disadvantages: (1) the safety of solvents like N-methyl-2-pyrrolidone (NMP), used to formulate these systems is questionable and not well documented, (2) the injection of these liquid implant systems and their subsequent solidification produce non-uniform matrix implants having variable consistency and geometry, and (3) due to formation of matrix implants having inconsistent texture, shape and size, the drug release from them is likely to be variable and unpredictable.

In the present paper, a novel method of *in situ* preparation of PLGA microspheres is described. The process of microsphere formation is based on the principle of phase separation (coacervation). This method overcomes the problems faced by the above systems by forming PLGA microglobules ("premicrospheres" or "embryonic microspheres") (Fig. 1). These microglobules on coming in contact with water, harden to form discrete PLGA microspheres (*in situ* formed microspheres) (Fig. 1). We describe here the composition, rationale, and optimization of this NDDS using a model heme protein, cytochrome *c*. The influence of various formulation variables such as the PLGA concentration and type and the substitution of the continuous phase by a fresh Oil Phase 2, on the characteristics of this system were evaluated. The reproducibility of the characteristics of the NDDS were investigated. Also, the effect of the formulation, process, and storage (15 days/4° C) conditions on the stability of the

NDDS and the physical stability of the encapsulated cytochrome *c* were determined.

MATERIALS

All the chemicals/reagents were used as purchased. Polyvinyl alcohol (PVA) (87.0-89.0% partially hydrolyzed, molecular weight: 11,000-31,000) from J. T. Baker (Phillipsburg, NJ), sodium azide and bovine heart cytochrome *c* (molecular weight: 12,327) from Sigma Chemical Co. (St. Louis, MO); PLGA RESOMER grades RG 502 H and RG 503 H from Boehringer Ingelheim (Ingelheim, Germany); triacetin from Eastman Fine Chemicals (Rochester, NY); polyethylene glycol 400 (PEG 400) from Union Carbide Corporation (Danbury, CT); miglyol 812 from Hüls America, Inc. (Piscataway, NJ); Span 80 and Tween 80 from I.C.I. (Wilmington, DE); DCM, n-hexane, and 2-propanol from Fisher Scientific (Fair Lawn, NJ).

METHODS

Preparation of injectable PLGA microglobules and subsequent *in situ* formation of microspheres

PLGA was dissolved in triacetin by magnetic stirring and heating to approximately 65° C in a glass vial. The solution was cooled under ambient condition and to it was added a solution of cytochrome *c* in PEG 400. This mixture was magnetically stirred for 5 min followed by addition of Tween 80 and further stirring for 5 min to form Oil Phase 1. Span 80 was dissolved in miglyol 812 in a glass vial to form Oil Phase 2. Oil Phase 1 was added dropwise to Oil Phase 2 with continuous homogenization at 10,000 rpm, using a Biohomogenizer (Biospec Products Inc., Bartlesville, OK) for a period of 4 min, to form the NDDS [injectable dispersion of PLGA microglobules in a continuous phase consisting of triacetin, PEG 400 and miglyol (and stabilized by Tween 80 and Span 80)]. The same procedure was also repeated to produce blank PLGA microglobules.

The ability of the NDDS to form microspheres *in situ* was performed in an aqueous phosphate buffer. The NDDS in 1 ml quantity was injected in 10 ml of 10 mM, 7.4 pH aqueous phosphate buffer contained in a 20 ml capacity glass vial. The phosphate buffer also contained 0.02% w/v PVA as an anti-adherent and 0.02% w/v sodium azide as a preservative (bacteriostatic). The above method was also repeated to obtain blank *in situ* formed PLGA microspheres.

(1) *In vitro* cytochrome *c* release

The glass vials containing the *in situ* formed microspheres were subjected to agitation at 100 rpm at 37° C in a G24 Environmental Incubator Shaker from New Brunswick Scientific Co., Inc. (Edison, NJ) (This step was also followed for blank *in situ* formed PLGA microspheres). Samples in 1 ml aliquot from each vial were withdrawn at predetermined time intervals using a 0.5 µm filter (Millipore Products Division, Bedford, MA) assisted 18G1 needle/1 cc plastic syringe assembly (Beckton Dickinson & Co., Rutherford, NJ). The vials were replenished with 1 ml of fresh aqueous phosphate buffer by passing it through the same filter assisted needle/syringe assembly. The amount of cytochrome *c* released from the *in situ* formed microspheres into the aqueous phosphate buffer was quantitatively determined by its absorbance at 409 nm using a UV-Vis Spectrophotometer from Varian (Varian Australia Pty. Ltd.). A standard concentration curve was used to determine the concentration of cytochrome *c* in the sample vials. The results were reported as mean of two experiments and for each experiment the mean of three sample analysis were determined.

(2) pH change determination

The pH change due to erosion of the PLGA copolymer and subsequent formation of lactic and glycolic acids was determined using a pH meter from Orion Research, Inc. (Cambridge, MA) and an attached microelectrode from Fisher Scientific. The vials containing the *in situ* formed microspheres were subjected to agitation at 100 rpm at 37° C and the pH measured at predetermined time intervals. The results were reported as mean of two experiments and for each experiment the mean of three sample analysis were determined.

(3) Optical microscopy

The transformation of the PLGA microglobules into *in situ* microspheres was documented using an Aristomet light microscope (Leitz, Germany) attached to a Kodak Megaplug camera. Samples representing different stages of transformation were observed using color optics and the micrographs developed using the Photoshot software from Adobe Systems, Inc. (San Jose, CA).

Extraction of the encapsulated cytochrome *c* from *in situ* formed PLGA microspheres

The vials containing the *in situ* formed microspheres were transferred to plastic centrifuging tubes, which were then centrifuged at 10,000 rpm for 10 min using a Hermle Z382K centrifuging machine from Denville Scientific, Inc. (Metuchen, NJ). The PLGA microspheres were washed twice, each time with 5 ml of n-hexane:2-propanol (1:1). This was then followed by washing with 5 ml of distilled water (twice). The tubes were then dried for 24 hr in a 282A Isotemp vacuum oven from Fisher Scientific and the weight of the dried PLGA microspheres determined. This was followed by addition of 5 ml of DCM to the tubes to dissolve the PLGA copolymer and subsequent extraction of the cytochrome *c* into 5 ml of distilled water. The aqueous phase containing the recovered cytochrome *c* was then subjected to percentage encapsulation efficiency study and circular dichroism (CD) spectroscopy.

The blank *in situ* formed PLGA microspheres were dried as above and their weights evaluated at various predetermined time intervals.

(1) Percentage cytochrome *c* encapsulation efficiency

The amount of cytochrome *c* in the aqueous phase was quantitatively determined by its absorbance at 409 nm. The percentage encapsulation efficiency was calculated by relating the practical cytochrome *c* entrapment to the theoretical cytochrome *c* entrapment,

Practical cytochrome *c* entrapment (% w/w)/Theoretical cytochrome *c* entrapment
(% w/w) x 100

The results were reported as mean of two experiments and for each experiment the mean of three sample analysis were determined.

(2) CD spectroscopy

The physical stability of the encapsulated cytochrome *c* was investigated by CD spectroscopy. The CD spectra were measured using a J-710 spectropolarimeter from Jasco, Inc. (Easton, MD). The conditions of analysis were as follows: pathlength, 0.1 cm (quartz cell); scan speed, 50 nm/min; step resolution, 1 nm/data; sensitivity, 50 mdeg; band width, 1 nm; wavelength range, 190-250 nm (far UV). For each sample an average of two scans were recorded using an attached SPL-430A X-Y plotter from Sekonic Company Ltd. (Japan).

(3) PLGA mass loss study

The blank *in situ* formed PLGA microspheres were evaluated for mass loss to study PLGA erosion by the gravimetry method. The percentage mass loss was calculated using the equation,

$$M_1 - M_2/M_1 \times 100$$

M_1 = weight in grams of the *in situ* formed PLGA microspheres; M_2 = weight in grams of the *in situ* formed PLGA microspheres at predetermined time intervals. The results were reported as mean of two experiments and for each experiment the mean of three sample analysis were determined.

Isolation of the *in situ* formed PLGA microspheres

The vials containing the *in situ* formed PLGA microspheres were subjected to vacuum filtering using a 30 μ m Whatman filter paper. This was followed by washing and drying of the microspheres as above. The isolated PLGA microsphere powder was then subjected to differential scanning calorimetry (DSC), particle size determination, and scanning electron microscopy (SEM) studies.

(1) DSC study

An SII differential scanning calorimeter from Seiko Instruments (Horsham, PA) was used for the thermal analysis. All the samples in approximately 3-7 mg quantity were weighed in standard aluminum pans which

were then sealed. A pin-hole was made in the center of the caps to allow escape of moisture during scanning. Thermograms were obtained at a scanning rate of 10° C/min and a temperature range of 22 to 180° C. The heating chamber was continuously purged with nitrogen gas at a rate of 50 ml/min. An attached 7550A graphics plotter (Hewlett-Packard Company) recorded the DSC profiles of the PLGA copolymer and the isolated *in situ* formed PLGA microspheres.

(2) Particle size determination study

The particle size of the isolated PLGA microspheres was determined using a Master Sizer X from Malvern Instruments, Inc. (Southborough, MA). The microspheres were first suspended in 100 ml of distilled water (containing 1% Tween 80) and subjected to sonication for 30 seconds and vortex mixing for 10 seconds before analysis. The volume average particle size of three scans for each sample was reported.

(3) SEM study

The topological and morphological characteristics of the *in situ* formed PLGA microspheres were documented by SEM. The isolated microspheres were mounted on metal stubs with double sided tapes and sputter coated with gold for 90 sec at 15 mAmp. These were then viewed under a JEOL JSM-840A scanning microscope (Peabody, MA) using 3 kV.

RESULTS AND DISCUSSION

Transformation of the PLGA microglobules into microspheres

Figure 2 depicts the optical micrographs of the different stages of conversion of a PLGA microglobule into microsphere. The microglobule consists of a rubbery PLGA matrix (Figure 2a). Figure 2b (8 min) illustrates an incompletely formed PLGA microsphere. The vehicles associated with the microglobules have been incompletely extracted in water, leading to formation of an intermediate and transient stage between microglobule and microsphere. Figure 2c (17 min) shows the final *in situ* formed PLGA microsphere. The extraction of vehicles in water is complete, resulting into formation of a hard and

opaque PLGA microsphere (containing cytochrome *c*). Due to the extraction of the vehicles, the microglobule subsequently shrinks in size during its transformation to the microsphere (Figure 2c).

NDDS composition, rationale, and optimization:

Oil Phase 1

(1) PLGA copolymer

Different manufacturers supply various grades of PLGA copolymer that are hydrophobic in nature and hence their hydrolytic degradation and subsequent drug release is delayed [27-30]. This delayed hydration of these polymers is due to “capping” or “blocking” of the terminal carboxylic acid end groups by esterification [27, 28]. Recently Boehringer Ingelheim has introduced new grades of PLGA in which the carboxylic acid end groups are not esterified and are free, thereby making these “uncapped” PLGA copolymers (RESOMER RG 50x H grade) more hydrophilic (or less hydrophobic) than the contemporary “capped” PLGA (RESOMER RG 50x grade) [27-30]. There is not much difference between RESOMER RG 50x and RESOMER RG 50x H grades with respect to certain physico-chemical properties like molecular weight, inherent viscosity, molar ratio, and glass transition temperature [30]. However, RESOMER RG 50x H grade being more hydrophilic, exhibits relatively rapid water uptake, hydrolysis, and subsequent drug release with no ‘lag time’ as compared to the RESOMER RG 50x grade [27-30].

Our goal was to achieve a rapid drug release from the final *in situ* formed microspheres and hence we selected the “uncapped” PLGA copolymer grade. The protein release was also dependent on the polymer molecular weight and its composition, i.e. lactide:glycolide ratio [4]. PLGA copolymers having lactide:glycolide ratio of 50:50 have been known to exhibit the fastest hydrolytic degradation [3]. Also for PLGA copolymer having similar composition, low molecular weight polymers are more hydrophilic and thus more rapidly eroding than the high molecular weight grades [3, 4]. We therefore selected the

“uncapped”, low molecular weight, more hydrophilic RESOMER RG 502 H grade for this invention.

The PLGA matrix should be strong enough to prevent leakage of the microglobule content. It should also maintain the integrity and shape of the microglobules and should not rupture or deform on redispersion of microglobules back in the continuous phase on gentle agitation. PLGA was dissolved in triacetin by heating to approximately 65° C. The concentration of PLGA used was found to be critical in this process and was optimized based on the PLGA/triacetin ratio and was in the range of 0.05 to 0.22. Preliminary studies involving higher PLGA/triacetin ratio (> 0.22) produced a sticky, coagulated PLGA mass (a non-microglobule product). In some cases, it even produced PLGA solutions that were too viscous to handle. Conversely, a low PLGA/triacetin ratio in these studies (< 0.05) resulted in poor entrapment and rapid leakage of the microglobule contents.

(2) Triacetin

Triacetin was selected to solubilize the PLGA polymer. Triacetin not only acts as a solvent, but also as a plasticizer for the copolymer, as evident from the DSC profiles in Fig. 3. The microencapsulation process resulted in a decrease in the glass transition temperature (T_g) of the PLGA copolymer by 4° C (Fig. 3). This decrease in T_g might affect the hydrolytic degradation (erosion) of the PLGA matrix and the subsequent release of the drug from it [31]. The concentration of triacetin plays an important role in this novel microencapsulation process and was optimized based on the PLGA/triacetin ratio.

(3) PEG 400

PEG 400 was employed as the second vehicle in the formulation, and it acted as a solvent for cytochrome *c*. The concentration of PEG 400 used in the formulation was of great significance and was determined based on the concentration of the PLGA-triacetin solution (i.e. PLGA-triacetin/PEG 400 ratio); it was optimized in the range of 1.6 to 8.0. Initial studies involving absence or very low PEG 400 concentrations (ratio > 8.0), produced a sticky and adhesive

PLGA mass (a non-microglobule product). Also, higher concentrations of PEG 400 (ratio < 1.6) produced poor quality PLGA microglobules.

(4) Tween 80

When the non-aqueous dispersion comes in contact with water, Tween 80 (HLB = 15) facilitated the maintenance of the PLGA microglobule spherical shape and also allowed efficient extraction of the vehicles in water. This resulted in hardening of PLGA microglobules into discrete and deaggregated microspheres which were then well dispersed in water. Experiments involving no Tween 80, yielded PLGA microglobules which poorly dispersed on coming in contact with water and they hardened into an agglomerated PLGA mass (a non-microsphere product).

Oil Phase 2

(1) Miglyol 812

A fractionated coconut oil, miglyol 812 (C₈:C₁₀ mixed triglycerides) was incorporated as the third vehicle for this novel microencapsulation process and it constituted the bulk of the continuous phase. The concentration of miglyol 812, was determined based on the miglyol 812/Oil Phase 1 ratio and was optimized in the range of 3.56 - 4.92. High concentration of miglyol 812 ensured complete extraction of triacetin and much of PEG 400. Also, miglyol 812 builds up the body of the dispersion and facilitates uniform redispersion of the PLGA microglobules (settled at the base of the container) back in the continuous phase on gentle agitation.

(2) Span 80

Span 80 (HLB = 4.3) prevented the PLGA microglobules from adhering to each other. Studies involving absence of Span 80 resulted in formation of a sticky and agglomerated PLGA mass (a non-microglobule product).

Percentage cytochrome c encapsulation efficiency and particle size studies

As depicted in Figure 4, increasing the PLGA concentration increased the percentage encapsulation efficiency of cytochrome *c*. The transportation of the cytochrome *c* to the aqueous buffer during the hardening of the PLGA

microglobules, leads to loss of cytochrome *c*. Extraction of the vehicles associated with the microglobules eventually leads to their hardening into microspheres, causing cessation of this transport. Increase in the PLGA concentration of the Oil Phase 1, would result in faster hardening of the PLGA microglobules. Also, higher viscosity of the Oil Phase 1 (due to higher PLGA loading) would decrease the loss transport of cytochrome *c* and cause enhanced percentage encapsulation efficiencies (Figure 4).

There was an increase in the microsphere size with increase in PLGA concentration (Figure 4). A similar result has also been reported by other groups. Higher PLGA concentration produces higher viscosity Oil Phase 1, which leads to a less effective coacervation process. As a result, larger PLGA microglobules are produced which subsequently get transformed to larger microspheres.

In vitro release of cytochrome *c* from the *in situ* formed PLGA microspheres

As shown in Figure 5, the *in situ* formed microspheres from the PLGA microglobules exhibit a controlled release of cytochrome *c*. As reported by other groups [4, 11, 13, 17], we suspect that the drug release from these microspheres is a combination of the following two mechanisms: diffusion through the microsphere matrix and its hydrolytic degradation (which is controlled by factors like PLGA molecular weight, crystallinity, and lactide:glycolide ratio). The release of drug from these microspheres is associated with formation of pores and they increase in number and size as the PLGA degradation proceeds, resulting in subsequent faster release rates of the drug [4]. The burst effect (amount of cytochrome *c* released by the end of day 1) is attributed mainly to the unencapsulated cytochrome *c*.

(1) Effect of PLGA concentration

A strong burst effect was observed for the microspheres prepared with lowest PLGA concentration (Figure 6). This is probably due to diffusion of surface-localized cytochrome *c* and also due to high concentration of unencapsulated cytochrome *c*, as a result of low percentage encapsulation efficiency (Figure 4). The smaller size of these microspheres resulting in a higher

surface area, as compared to the microspheres prepared using higher PLGA concentrations, could also account for this effect. Depending upon the PLGA/triacetin ratio, microglobules having different consistency are produced. Microspheres from the lowest PLGA/triacetin ratio (0.05), have a less dense and rigid PLGA matrix and hence they exhibit the fastest drug release. The opposite is true for the microspheres prepared from the highest PLGA/triacetin ratio (0.18) (Figure 6).

(2) Effect of PLGA type

The drug release profile of the *in situ* formed PLGA microspheres prepared from RESOMER RG 502 H were compared with that of RG 503 H (Figure 7). RG 503 H has a relatively higher molecular weight and is thus relatively more hydrophobic than the RG 502 H grade. Hence RG 503 H undergoes relatively slower hydrolytic degradation, thereby exhibiting relatively lower burst and prolonged release of cytochrome *c* as compared to RG 502 H (Figure 7).

(3) Effect of replacement of the continuous phase by a fresh Oil Phase 2

After the settling of the PLGA microglobules at the bottom of the glass vial, the continuous phase consisting of triacetin, miglyol 812, and PEG 400 was decanted and a fresh Oil Phase 2 was added to the PLGA microglobules. This new NDDS was then compared to the original NDDS with respect to cytochrome *c* release profile (Figure 8). As expected, the *in situ* formed microspheres produced from the new NDDS exhibited relatively low burst and retarded cytochrome *c* release as compared to those produced from the original NDDS. This could be due to absence of the unencapsulated cytochrome *c* in the fresh Oil Phase 2.

PLGA erosion study

(1) pH change study

The difference in the PLGA erosion with respect to its loading was determined by monitoring the pH changes, since random hydrolytic ester cleavage produces polymer fragments with carboxylic acid end groups [32]. Figure 9a

shows the pH change of the 10 mM, 7.4 pH aqueous phosphate buffer (subsequent to *in situ* formation of PLGA microspheres) as the function of incubation time. All the formulations showed a marked decrease in the pH of the phosphate buffer with increasing incubation period. The hydrogen ions liberated due to ionization of the carboxylic acid end groups of PLGA [32], overwhelm the buffering capacity of the 10 ml, 10 mM phosphate buffer leading to a decrease in its pH. The ionization process not only increases the permeability of the microspheres, but also facilitates autocatalysis [32]. A series of these events are responsible for the controlled release of cytochrome *c* from the *in situ* formed microspheres. The use of relatively more hydrophilic, uncapped PLGA grade (RESOMER RG 502 H) could be additionally responsible for the observed decrease in the pH. Microspheres prepared from the highest PLGA concentration (ratio of 0.18) are relatively more hydrophobic than those prepared from the lowest PLGA concentration (ratio of 0.05) and hence they exhibited the slowest pH change of the phosphate buffer (Figure 9a).

(2) Mass loss study

The loss of mass of the *in situ* formed PLGA microspheres with increasing incubation time also indicated the PLGA erosion of the *in situ* formed microspheres (Figure 9b). This proved that they undergo extensive degradation to produce water soluble monomers and oligomers, resulting in their weight loss [32]. Highest PLGA concentration microspheres, since being more hydrophobic showed the slowest degradation as compared to those prepared using the lowest PLGA concentration (Figure 9b).

Reproducibility of the characteristics of the NDDS

In due course of time, the dispersed microglobules separate from the continuous phase and tend to adhere and settle at the base of the container. It is imperative that the NDDS exhibit no change in its characteristics on redispersion by gentle agitation. Cytochrome *c* release profile of the *in situ* formed microspheres produced from the original NDDS and a redispersed NDDS after 1 hr is shown in Figure 10. As shown, the release profile of cytochrome *c* was

relatively unchanged. Also there was not much difference in the particle size and the percentage encapsulation efficiency of the *in situ* formed microspheres produced from the original and redispersed PLGA microglobules.

Stability studies of the NDDS

It is necessary for protein formulations to be stored at appropriate conditions, in order to maintain the stability of the encapsulated protein drug. Hence the NDDS was stored at 4° C for a period of 15 days and the characteristics of the system then evaluated after redispersion on gentle agitation. As depicted in Figure 11, the release profiles of the *in situ* formed microspheres produced from the original and stored PLGA microglobules are almost identical. Also there was not much difference in their particle sizes and the percentage encapsulation efficiencies. This confirmed that the characteristics of the NDDS were unaltered on their storage at 4° C for a period of 15 days.

It is of paramount importance to maintain the native protein conformation and integrity during the encapsulation process. The physical stability of cytochrome *c* was evaluated by CD spectroscopy. The CD profile of cytochrome *c* in the far UV range provided the secondary structure of the protein. As evident from Figures 12a and 12b the ellipticity around 222 nm was not changed by the formulation/process and the 15 days/4° C storage conditions respectively. This suggested that the protein maintained its α -helical structure and thus its physical stability.

SEM study of the *in situ* formed PLGA microspheres

Figures 13a and 13b reveal the topological and morphological characteristics of the *in situ* formed PLGA microspheres as observed using SEM. As shown, the microspheres are spherical and monodispersed in size (Figure 13a). Also their surface shows very little or no pore structures (Figure 13b). Thus this novel microencapsulation process tends to produce virtually non-porous microspheres, unlike the solvent evaporation method which yields microspheres having porous surface structure. This could also explain the low burst effect

observed for the microspheres in our study and the drug released by the end of day one is the unencapsulated cytochrome *c*.

CONCLUSION

The composition, rationale, and optimization of the NDDS is described here. Various formulation variables such as the PLGA concentration and type and the substitution of the continuous phase by a fresh Oil Phase 2 influenced the characteristics of this system. The NDDS was reproducible and its characteristics were not affected by the storage condition. The formulation, process, and storage conditions did not affect the physical stability of the encapsulated cytochrome *c*. The SEM study of these microspheres revealed a non-porous surface structure. This novel microencapsulation process overcomes some of the disadvantages associated with other methods by: (1) excluding the use of unacceptable organic solvents like DCM or NMP and using acceptable vehicle mixture instead to prepare biodegradable PLGA microspheres, (2) forming drug containing PLGA microglobules ("premicrospheres" or "embryonic microspheres") which could be considered as precursors to the final microsphere product; these on coming in contact with water harden to form discrete PLGA microspheres which subsequently exhibit non-variable, predictable, and controlled drug release profile, and (3) precluding the need for reconstitution of the PLGA microspheres before their administration. Thus, this novel microencapsulation method could be viewed as a modified coacervation process, wherein cytochrome *c* containing microglobules are converted to microspheres *in situ*. This process can also yield microspheres in an isolated, powder form.

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Figure 1-Schematic representation of the novel *in situ* PLGA microsphere formation process

Figure 2-Transformation of the PLGA microglobule into microsphere

(a)

(b)

(c)

Figure 3-DSC profiles of the PLGA copolymer and the *in situ* formed PLGA microsphere

Figure 4-Effect of PLGA concentration on the particle size (μm) and percentage cytochrome *c* encapsulation efficiency of the *in situ* formed microspheres (PLGA concentration expressed as different ratios of PLGA/triacetin)

Figure 5-*In vitro* release of cytochrome *c* from *in situ* formed PLGA microspheres
(PLGA concentration expressed as the ratio of PLGA/triacetin is 0.11)

Figure 6-Controlled release of cytochrome *c* from *in situ* formed PLGA microspheres prepared from NDDS containing different PLGA concentrations (PLGA concentration expressed as different ratios of PLGA/triacetin)

Figure 7-Controlled release of cytochrome *c* from *in situ* formed PLGA microspheres prepared from NDDS containing different PLGA types

Figure 8-Controlled release of cytochrome *c* from *in situ* formed PLGA microspheres prepared from the original and modified NDDS (replacing the continuous phase by fresh Oil Phase 2)

Figure 9a-pH change of *in situ* formed PLGA microspheres prepared from NDDS containing different PLGA concentrations (PLGA concentration expressed as different ratios of PLGA/triacetin)

Figure 9b-Mass loss (erosion) of *in situ* formed PLGA microspheres prepared from NDDS containing different PLGA concentrations (PLGA concentration expressed as different ratios of PLGA/triacetin)

Figure 10-Controlled release of cytochrome *c* from *in situ* formed PLGA microspheres prepared from the original NDDS and the same NDDS on gentle agitation after 1 hr (reproducibility of the characteristics of the NDDS)

Figure 11-Controlled release of cytochrome *c* from *in situ* formed PLGA microspheres prepared from the original NDDS and the same NDDS after storage for 15 days at 4° C (stability study of the NDDS)

Figure 12a-Effect of formulation and process conditions on the physical stability of the encapsulated cytochrome *c*

Figure 12b-Effect of 15 days/4° C storage condition on the physical stability of the encapsulated cytochrome *c*

Figure 13a-SEM picture of the *in situ* formed PLGA microspheres (low magnification)

Figure 13b-SEM picture of the *in situ* formed PLGA microspheres (high magnification)

MANUSCRIPT III

CONTROLLED RELEASE OF DRUGS FROM INJECTABLE *IN SITU*
FORMED BIODEGRADABLE PLGA MICROSPHERES: EFFECT
OF VARIOUS FORMULATION VARIABLES

ABSTRACT

An *in situ* method for the preparation of injectable biodegradable microspheres from the copolymer, poly(lactide-*co*-glycolide) (PLGA) for the delivery of micromolecule and macromolecules such as proteins is described. The delivery system is the same as described earlier (1) and it involves a stable dispersion of PLGA microglobules (“premicrospheres” or “embryonic microspheres”) in an acceptable vehicle mixture (continuous phase). A solution of PLGA, triacetin, drug, PEG 400, and Tween 80 (Oil Phase 1) are added dropwise with continuous homogenization to miglyol 812-Span 80 solution (Oil Phase 2), thereby inducing phase separation (coacervation) of PLGA and forming PLGA microglobules (containing the drug) dispersed in the continuous phase. This novel drug delivery system (NDDS) on injection comes in contact with water from aqueous buffer or physiological fluid, thereby hardening the microglobules into solid matrix type microparticles entrapping the drug (*in situ* formed microspheres) (1). The drug is then released from these microspheres in a controlled fashion. The concentration of PEG 400, the encapsulated drug, and the added hydrophilic excipient (mannitol) affected the characteristics of this NDDS. The characteristics were also influenced by the types of encapsulated drug and the vehicles added to the system. The physical stability of the encapsulated protein was unaffected by the formulation, process, and the 15 days/4° C storage conditions.

INTRODUCTION

There is a particular interest in controlled delivery of macromolecules such as peptides and proteins through PLGA (the copolymer of lactic and glycolic acids) microspheres and a wealth of literature has been generated on this issue (2-18). PLGAs have shown to be biocompatible and they degrade to toxicologically acceptable lactic and glycolic acids that are eventually eliminated from the body (2). Also, they have been approved by the FDA as controlled drug release microspheres (4, 5).

The NDDS is the same as described earlier (1) (Figure 1) and it involves a stable dispersion of PLGA microglobules ("premicrospheres" or "embryonic microspheres") in a continuous phase consisting of acceptable vehicle mixture. This on injection, comes in contact with water from aqueous buffer or physiological fluid, thereby hardening the microglobules into solid matrix type microparticles entrapping the drug (*in situ* formed microspheres) (1). The drug is then released from these microspheres in a controlled fashion. This novel process overcomes some of the disadvantages associated with other methods by: (i) replacing the unacceptable organic solvents by an acceptable vehicle mixture to prepare biodegradable PLGA microspheres, (ii) producing drug containing PLGA microglobules ("premicrospheres" or "embryonic microspheres") which could be considered as precursors to the final microsphere product; these on coming in contact with water harden to form discreet PLGA microspheres which subsequently exhibit non-variable, predictable, and controlled drug release profile, and (iii) avoiding the need for reconstitution of the PLGA microspheres before their administration (1).

In our previous study we had described the composition, rationale, and optimization of the NDDS (1). Various formulation variables such as the PLGA loading and type and the substitution of the continuous phase by a fresh Oil Phase 2 influenced the characteristics of this system. The NDDS was reproducible and its characteristics were not affected by the storage condition (1). Circular dichroism (CD) spectroscopy revealed that the formulation, process, and the

storage conditions did not adversely affect the physical stability of the encapsulated protein, cytochrome *c*. The scanning electron microscopy study of these microspheres revealed spherical and monodispersed particles having non-porous surface structures (1).

In this study, we have investigated the effect of the following formulation variables on the characteristics of the NDDS: (i) the concentrations of PEG 400, the encapsulated drug, and the hydrophilic excipient (mannitol) and (ii) the types of encapsulated drug and the vehicles. Also, the effect of formulation, process, and storage (15 days/4° C) conditions on the stability of the encapsulated protein was evaluated.

MATERIALS

All the chemicals/reagents were used as purchased. Polyvinyl alcohol (PVA) (87.0-89.0% partially hydrolyzed, molecular weight: 11,000-31,000) from J. T. Baker (Phillipsburg, NJ); sodium azide, bovine heart cytochrome *c* (molecular weight: 12, 327), and heart skeletal muscle myoglobin (molecular weight: 16, 950) from Sigma Chemical Co. (St. Louis, MO); red D&C #33 dye (red dye) from Pylam Products Corp. (Garden City, NY); PLGA RESOMER grade RG 502 H from Boehringer Ingelheim (Ingelheim, Germany); triacetin from Eastman Fine Chemicals (Rochester, NY); triethyl citrate from Morflex Chemical Co. (Greensboro, NC); polyethylene glycol 400 (PEG 400) from Union Carbide Corp. (Danbury, CT); miglyol 812 from Hüls America, Inc. (Piscataway, NJ); soybean oil from Welch, Holme & Clark Co., Inc. (Newark, NJ); mannitol from Roquette America, Inc. (Keokuk, IA); Span 80 and Tween 80 from I.C.I. (Wilmington, DE); methylene chloride, n-hexane, and 2-propanol from Fisher Scientific (Fair Lawn, NJ).

METHODS

NDDS formation

PLGA was dissolved in triacetin by magnetic stirring and heating to approximately 65° C in a glass vial. The solution was cooled under ambient condition and to it was added a solution of cytochrome *c* in PEG 400. This mixture was magnetically stirred for 5 min followed by addition of Tween 80 and further stirring for 5 min to form Oil Phase 1. Span 80 was dissolved in miglyol 812 in a glass vial to form Oil Phase 2. Oil Phase 1 was added dropwise to Oil Phase 2 with continuous homogenization at 10,000 rpm, using a Biohomogenizer (Biospec Products Inc., Bartlesville, OK) for a period of 4 min, to form the NDDS [injectable dispersion of PLGA microglobules in a continuous phase consisting of triacetin, PEG 400 and miglyol (and stabilized by Tween 80 and Span 80)].

Optical microscopy

The transformation of the PLGA microglobules into *in situ* microspheres was documented using an Aristomet light microscope (Leitz, Germany) attached to a Kodak Megaplug camera. Samples representing different stages of the transformation were observed using color optics and the micrographs developed using the Photoshop software from Adobe Systems, Inc. (San Jose, CA).

In vitro drug release from the in situ formed PLGA microspheres

The NDDS in 1 ml quantity was injected in 10 ml of 10 mM, 7.4 pH aqueous phosphate buffer contained in a 20 ml capacity glass vial to *in situ* form microspheres. The phosphate buffer also contained 0.02% w/v PVA as an anti-adherent and 0.02% w/v sodium azide as a preservative (bacteriostatic). The glass vials containing the *in situ* formed microspheres were subjected to agitation at 100 rpm at 37° C in a G24 Environmental Incubator Shaker from New Brunswick Scientific Co., Inc. (Edison, NJ). Samples in 1 ml aliquot from each vial were withdrawn at predetermined time intervals using a 0.5 µm filter (Millipore Products Division, Bedford, MA) assisted 18G1 needle/1 cc plastic syringe assembly (Beckton Dickinson & Co., Rutherford, NJ). The vials were replenished with 1 ml of fresh aqueous phosphate buffer by passing it through the same filter

assisted needle/syringe assembly. The amount of drug released from the *in situ* formed microspheres into the aqueous phosphate buffer was quantitatively determined using a UV-Vis Spectrophotometer from Varian (Varian Australia Pty. Ltd.). The wave length used for the analysis of the red dye was 530 nm while the proteins, cytochrome *c* and myoglobin were analyzed at 409 nm. A standard concentration curve was used to determine the concentration of the drug in the sample vials. The results were reported as mean of two experiments and for each experiment the mean of three sample analysis were determined.

Percentage encapsulation efficiency and CD spectroscopy studies

The vials containing the *in situ* formed microspheres were transferred to plastic centrifuging tubes, which were then centrifuged at 10, 000 rpm for 10 min using a Hermle Z382K centrifuging machine from Denville Scientific, Inc. (Metuchen, NJ). The PLGA microspheres were washed twice, each time with 5 ml of n-hexane:2-propanol (1:1). This was then followed by washing with 5 ml of distilled water (twice). The tubes were then dried for 24 hr in a 282A Isotemp vacuum oven from Fisher Scientific and the weight of the dried PLGA microspheres determined. This was followed by addition of 5 ml of methylene chloride to the tubes to dissolve the PLGA copolymer and subsequent extraction of the cytochrome *c* into 5 ml of distilled water. This process was also repeated for myoglobin-encapsulated microspheres.

The amount of cytochrome *c* in the aqueous phase was quantitatively determined by its absorbance at 409 nm. The percentage encapsulation efficiency was calculated by relating the practical cytochrome *c* entrapment to the theoretical cytochrome *c* entrapment,

$$\frac{\text{Practical cytochrome } c \text{ entrapment (\% w/w)}}{\text{Theoretical cytochrome } c \text{ entrapment (\% w/w)}} \times 100$$

The results were reported as mean of two experiments and for each experiment the mean of three sample analysis were determined.

The aqueous phase containing the extracted myoglobin was subjected to CD spectroscopy using a J-710 spectropolarimeter from Jasco, Inc. (Easton, MD). The conditions of analysis were as follows: pathlength, 0.1 cm (quartz cell); scan speed, 50 nm/min; step resolution, 1 nm/data; sensitivity, 50 mdeg; band width, 1 nm; wavelength range, 190-250 nm (far UV). For each sample an average of two scans were recorded using an attached SPL-430A X-Y plotter from Sekonic Company Ltd. (Japan).

RESULTS AND DISCUSSION

In situ formation of PLGA microspheres

In our previous paper, we had shown by optical microscopy the process of transformation of the PLGA microglobules into microspheres (1). This transformation occurred in approximately 17 min (1). Figure 2a shows the rubbery PLGA microglobules (containing the red dye) while Figure 2b shows the hard and opaque *in situ* formed microspheres (17 min after injection of the microglobules in the aqueous phosphate buffer). The microglobules on coming in contact with water harden to form solid matrix type microparticles entrapping the drug (*in situ* formed microspheres) (1). Also, due to the extraction of the vehicles associated with the microglobules by water, they subsequently shrink in size during their transformation to microspheres (Figures 2a and 2b) (1).

In vitro release of drugs from the *in situ* formed PLGA microspheres

As discussed in our previous paper, the *in situ* formed microspheres from the PLGA microglobules exhibited a controlled cytochrome *c* release from few days upto two weeks, depending upon various formulation variables (1). The burst effect (amount of cytochrome *c* released by the end of day 1) was mainly due to the unencapsulated cytochrome *c* (1).

(1) Effect of encapsulating different drug type

Proteins are too large to diffuse through the polymer matrix and their release occurs via diffusion through the interconnecting channels and pores

formed by them in the matrix (5, 19). The proteins are also released by the hydrolytic degradation (erosion) of the matrix (5, 19). Kissel *et al.* have reported a decrease in the release of the encapsulated proteins with increase in their molecular weights (20). Figure 3 shows the *in vitro* drug release profile of the red dye, cytochrome *c* and myoglobin from the *in situ* formed PLGA microspheres. As shown, the release of myoglobin (molecular weight: 16, 950) is slightly slower than cytochrome *c* (molecular weight: 12, 327). Also, the red dye (a micromolecule) is released rapidly from the PLGA matrix due to its faster diffusion from the matrix (Figure 3).

(2) Effect of PEG 400 and drug loading

The concentration of PEG 400 used in the formulation was of great significance and was determined based on the concentration of the PLGA-triacetin solution (i.e. PLGA-triacetin/PEG 400 ratio). The PEG 400 concentration was optimized in the range of 1.59 to 7.97 (1). Decrease in the concentration of PEG 400 (ratio of 6.03) produces a concentrated PEG 400-cytochrome *c* solution. Thus more drug is available to be encapsulated by the PLGA microglobules, resulting in higher percentage cytochrome *c* encapsulation efficiency (Figure 4a). This increased concentration of the encapsulated cytochrome *c* results in an increase in the interconnecting channels and pores in the PLGA matrix, leading to higher release rate and faster drug release profile (Figure 4a). An increase in the concentration of cytochrome *c* also produces a concentrated PEG 400-cytochrome *c* solution. Thus more drug is encapsulated by the PLGA microglobules, resulting in higher percentage cytochrome *c* encapsulation efficiencies and accelerated release profile (Figure 4b).

(3) Effect of addition of an hydrophilic excipient (mannitol)

Figure 5 shows the release of myoglobin in presence of the hydrophilic excipient, mannitol. Various groups have reported the effect of different additives/excipients on the drug release from the PLGA microparticles (5, 19, 21). Proteins are released from the PLGA matrix by diffusion from the interconnecting channels and pores formed by them in the matrix and by the hydrolytic

degradation (erosion) of the matrix. Addition of co-diffusants like mannitol imparts porosity to the PLGA matrix, thereby changing the matrix structure and providing increased diffusion through interconnecting channels for the release of the proteins. Thus, increase in the concentration of mannitol leads to subsequent higher protein release profile due to formation of highly porous PLGA matrix (Figure 5). We suspect that an increase in the mannitol concentration would lead to subsequent lower percentage encapsulation efficiencies, as mannitol would compete with myoglobin to be encapsulated by the PLGA microglobules.

(4) Effect of using different vehicles

The plasticizer, triacetin (solubility in water: 77.8 mg/ml) is relatively more hydrophilic than triethyl citrate (solubility in water: 55.4 mg/ml) (22). Thus triethyl citrate would cause relatively greater plasticization of the hydrophobic PLGA copolymer than triacetin. Hence microspheres prepared using triethyl citrate would undergo relatively enhanced hydrolytic degradation and subsequent faster release of the drug as compared to those formulated using triacetin (Figure 6a).

Soybean oil (mixed triglyceride of unsaturated and saturated fatty acids) is relatively more hydrophobic than miglyol 812 (mixed C₈-C₁₀ triglyceride of saturated fatty acids). Hence use of soybean oil produces relatively more hydrophobic PLGA microglobule dispersion than those formulated using miglyol 812, thereby delaying the dissolution of the released myoglobin. Thus microspheres prepared from NDDS containing soybean oil exhibit relatively retarded myoglobin release as compared to those produced from NDDS containing miglyol 812 (Figure 6b).

Stability of the encapsulated myoglobin

It is important to maintain the native protein conformation and its stability during the encapsulation process. In our previous paper, we had shown by CD spectroscopy that the formulation, process and the 15 days/4° C storage conditions did not adversely affect the physical stability of the encapsulated cytochrome *c*

(1). The CD profile of myoglobin in the far UV range (which provided the secondary structure of the protein) has been shown in Figures 7a and 7b. As evident from these figures, the ellipticity around 222 nm was unaffected by the formulation/process and the 15 days/4° C storage conditions. This suggested that the protein maintained its α -helical structure and hence its physical stability.

CONCLUSION

Controlled release of micromolecule and proteins from the *in situ* formed PLGA microspheres produced from a novel coacervation process is described here. The *in vitro* drug release was enhanced with decrease in the PEG 400 concentration and increase in the drug and mannitol concentration. The drug release was retarded with increase in the molecular weight of the encapsulated drug. Substitution of triacetin by triethyl citrate accelerated the drug release while replacing miglyol 812 with soybean oil slowed the drug release from the *in situ* formed microspheres. The stability of the encapsulated myoglobin was unaffected by the formulation, process, and the storage conditions. This novel microencapsulation process, besides producing microspheres *in situ*, also yielded microspheres in an isolated, powder form.

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Figure 1-Schematic representation of the novel *in situ* PLGA microsphere formation process

Figure 2-Transformation of the PLGA microglobules into microspheres

Figure 3-Controlled release of different drugs from *in situ* formed PLGA microspheres

Figure 4a-Effect of PEG 400 concentration on the controlled release and percentage encapsulation efficiency of cytochrome *c* (PEG 400 concentration expressed as different ratios of PLGA-triacetin/PEG 400)

Figure 4b-Effect of cytochrome *c* concentration on its controlled release and percentage encapsulation efficiency (cytochrome *c* concentration expressed as % w/w of PEG 400)

Figure 5-Controlled release of myoglobin from *in situ* formed PLGA microspheres prepared from NDDS containing different concentrations of the hydrophilic excipient, mannitol (mannitol concentration expressed as % w/w of myoglobin-PEG 400 solution)

Figure 6a-Controlled release of myoglobin from *in situ* formed PLGA microspheres prepared from NDDS containing different plasticizers

Figure 6b-Controlled release of myoglobin from *in situ* formed PLGA microspheres prepared from NDDS containing different oils

Figure 7a-Effect of formulation and process conditions on the physical stability of the encapsulated myoglobin

Figure 7b-Effect of 15 days/4° C storage condition on the physical stability of the encapsulated myoglobin

MANUSCRIPT IV

COMPARISON OF VARIOUS INJECTABLE PROTEIN LOADED
BIODEGRADABLE PLGA DEVICES: *IN SITU* FORMED
IMPLANT V/S *IN SITU* FORMED MICROSPHERES
V/S ISOLATED MICROSPHERES

ABSTRACT

Controlled delivery of a model protein, cytochrome *c* from various biodegradable devices prepared from the copolymer poly(lactide-*co*-glycolide) (PLGA) by a novel, modified microencapsulation process is described here. This method which did not involve any use of unacceptable organic solvents, but utilized acceptable vehicle mixture instead, produced *in situ* formed microspheres (Jain et. al., a,b). The modification of this novel process yielded different syringeable mixtures. These on injection, came in contact with water from aqueous buffer or physiological fluid and as a result, they hardened to form solid matrix type implant or microspheres (*in situ* formed implant or microspheres respectively) entrapping cytochrome *c*. This modified method can also be made to produce injectable, isolated microspheres. These devices exhibited different characteristics in terms of *in vitro* cytochrome *c* release profile, percentage cytochrome *c* encapsulation efficiency, and particle size. Thus this novel, modified microencapsulation process is versatile and it can produce various drug loaded injectable biodegradable PLGA devices having different characteristics.

INTRODUCTION

The copolymer of lactide and glycolide referred to as poly(lactide-co-glycolide) (PLGA) has been fabricated into various devices like microparticles, nanoparticles, implants, and films to carry a variety of drug classes (Jalil and Nixon, 1990; Tice and Tabibi, 1991; Wu, 1995a,b; Lewis, 1990; Heller, 1980; Heller, 1984; Heller, 1985; Tice and Cowsar, 1984; Arshady, 1991; Brannon-Peppas, 1995; Kitchell and Wise, 1985; Cohen et. al., 1994; Vert, 1996; Zhao and Leong, 1996; Eldridge et. al., 1993; Tice et. al., 1996). The drug release from the PLGA device is a combination of the following two mechanisms (Jalil and Nixon, 1990; Tice and Tabibi, 1991; Wu, 1995a,b): diffusion through the matrix and its hydrolytic degradation (which is controlled by factors like PLGA molecular weight, crystallinity, and lactide:glycolide ratio).

There is a particular interest in controlled delivery of macromolecules such as peptides and proteins through PLGA microspheres (Lewis, 1990). Although a wide variety of pharmacologically useful peptide and protein based drugs have been recently developed by genetic engineering, their therapeutic use is restricted due to certain disadvantages: (Wu, 1995b; Lewis, 1990): (1) on oral consumption they are subject to attack by the acidic and enzymatic environment in the stomach and the enzymes from the brush border membrane of the intestine, (2) their high molecular weight and size impede their effective transport across the gastrointestinal membranes, and (3) they have a short biological half-life and on injection they are quickly metabolized and eliminated. To achieve sustained blood levels of these drugs, minimize their denaturation or degradation, and to extend their biological half-life, their delivery by entrapment in PLGA devices has become an interesting approach (Wu, 1995b; Lewis, 1990). The release of proteins from these devices is associated with formation of pores and they increase in number and size as the PLGA degradation proceeds, resulting in subsequent faster release rates of the protein (Wu, 1995b).

Shah et al. (1993) and the researchers at Atrix Laboratories, Inc. (Fort Collins, CO) (Dunn et. al., 1995a,b; Frank et. al., 1994; Dunn et. al., 1994; Lowe

et. al., 1993; Dunn et. al., 1992; Tipton et. al., 1992; Dunn et. al., 1991) have described a novel implant system which is parenterally administered as a liquid and subsequently solidifies into a gel matrix (implant) *in situ*, from which the drug is released in a controlled manner. We have previously described the *in situ* formation of microspheres by a novel microencapsulation process, which was based on the principle of phase separation (coacervation) (Jain et. al., a,b). This method produced PLGA microglobules ("premicrospheres" or "embryonic microspheres") which on coming in contact with water, hardened to form discreet PLGA microspheres (*in situ* formed microspheres), releasing cytochrome *c* in a controlled fashion. Various formulation variables affected the characteristics of this system (Jain et. al. a,b). Also the formulation, process, and the 15 days/4° C storage conditions did not affect the physical stability of the encapsulated cytochrome *c*.

Here we report a modified version of the above novel microencapsulation process, which besides producing injectable *in situ* formed microspheres, can also be made to yield injectable *in situ* formed implant or isolated microspheres (Fig. 1). The heme protein, cytochrome *c* was encapsulated by these three devices and their characteristics with respect to *in vitro* cytochrome *c* release, percentage cytochrome *c* encapsulation efficiency, and particle size were compared.

MATERIALS

All the chemicals/reagents were used as purchased. Polyvinyl alcohol (PVA) (87.0-89.0% partially hydrolyzed, molecular weight: 11,000-31,000) from J. T. Baker (Phillipsburg, NJ); sodium azide and bovine heart cytochrome *c* (molecular weight: 12, 327) from Sigma Chemical Co. (St. Louis, MO); PLGA RESOMER grade RG 502 H from Boehringer Ingelheim (Ingelheim, Germany); triacetin from Eastman Fine Chemicals (Rochester, NY); polyethylene glycol 400 (PEG 400) from Union Carbide Corporation (Danbury, CT); miglyol 812 from Hüls America, Inc. (Piscataway, NJ); Span 80 and Tween 80 from I.C.I.

(Wilmington, DE); methylene chloride, n-hexane, and 2-propanol from Fisher Scientific (Fair Lawn, NJ).

METHODS

Preparation of injectable PLGA implant system

PLGA was dissolved in triacetin by magnetic stirring and heating to approximately 65° C in a glass vial. The solution was cooled under ambient condition and to it was added a solution of cytochrome *c* in PEG 400. This mixture was magnetically stirred for 5 min followed by additon of Tween 80 and further stirring for 5 min to form the injectable *in situ* implant system (Oil Phase 1). The ability of this system to form an implant *in situ* was performed in an aqueous phosphate buffer. The system in 1 ml quantity was injected in 10 ml of 10 mM, 7.4 pH aqueous phosphate buffer contained in a 20 ml capacity glass vial. The phosphate buffer also contained 0.02% w/v PVA as an anti-adherent and 0.02% w/v sodium azide as a preservative (bacteriostatic).

Preparation of injectable, stable dispersion of PLGA microglobules

Span 80 was dissolved in miglyol 812 in a glass vial to form Oil Phase 2. Oil Phase 1 was added dropwise to Oil Phase 2 with continuous homogenization at 10,000 rpm, using a Biohomogenizer (Biospec Products Inc., Bartlesville, OK) for a period of 4 min, to form the injectable, stable dispersion of PLGA microglobules [in a continuous phase consisting of triacetin, PEG 400 and miglyol (and stabilized by Tween 80 and Span 80)]. The ability of this system to form microspheres *in situ* was performed in an aqueous phosphate buffer. The system in 1 ml quantity was introduced in 10 ml of 10 mM, 7.4 pH aqueous phosphate buffer contained in a 20 ml capacity glass vial. The phosphate buffer contained PVA and sodium azide.

Preparation of injectable, isolated PLGA microspheres

The injectable dispersion of PLGA microglobules from above was added dropwise to a continuously stirred (700 rpm), 2 L 1% PVA solution to harden the microglobules into microspheres. These were then subjected to vacuum filtering using a 30 μm Whatman filter paper. The microspheres were then washed with 500 ml of distilled water. This was followed by washing twice with 100 ml of n-hexane:iso-propanol (1:1) and further washing with 100 ml of distilled water (twice). The isolated microspheres were finally dried for 24 hr in a 282A Isotemp vacuum oven from Fisher Scientific.

Optical microscopy

The various injectable biodegradable PLGA devices were documented using an Aristomet light microscope (Leitz, Germany) attached to a Kodak Megaplug camera. The devices were observed using color optics and the micrographs developed using the Photoshot software from Adobe Systems, Inc. (San Jose, CA).

In vitro cytochrome c release study

The glass vials containing the *in situ* formed devices were subjected to agitation at 100 rpm at 37° C in a G24 Environmental Incubator Shaker from New Brunswick Scientific Co., Inc. (Edison, NJ). The isolated microspheres were suspended in the phosphate buffer and agitated in a similar manner. Samples in 1 ml aliquot from each vial were withdrawn at predetermined time intervals using a 0.5 μm filter (Millipore Products Division, Bedford, MA) assisted 18G1 needle/1 cc plastic syringe assembly (Beckton Dickinson & Co., Rutherford, NJ). The vials were replenished with 1 ml of fresh aqueous phosphate buffer by passing it through the same filter assisted needle/syringe assembly. The amount of cytochrome *c* released from the devices into the aqueous phosphate buffer was quantitatively determined by its absorbance at 409 nm using a UV-Vis Spectrophotometer from Varian (Varian Australia Pty. Ltd.). A standard concentration curve was used to determine the concentration of cytochrome *c* in

the sample vials. The results were reported as mean of two experiments and for each experiment the mean of three sample analysis were determined.

Percentage cytochrome *c* encapsulation efficiency study

The vials containing the *in situ* formed devices were transferred to plastic centrifuging tubes, which were then centrifuged at 10,000 rpm for 10 min using a Hermle Z382K centrifuging machine from Denville Scientific, Inc. (Metuchen, NJ). The devices were washed twice, each time with 5 ml of n-hexane:2-propanol (1:1). This was then followed by washing with 5 ml of distilled water (twice). The tubes were then dried for 24 hr under vacuum and the weight of the dried PLGA microspheres determined. This was followed by addition of 5 ml of methylene chloride to the tubes to dissolve the PLGA copolymer and subsequent extraction of the cytochrome *c* into 5 ml of distilled water. This step was also followed for isolated microspheres. The amount of cytochrome *c* in the aqueous phase was quantitatively determined by its absorbance at 409 nm. The percentage encapsulation efficiency was calculated by relating the practical cytochrome *c* entrapment to the theoretical cytochrome *c* entrapment,

$$\frac{\text{Practical cytochrome } c \text{ entrapment (\% w/w)}}{\text{Theoretical cytochrome } c \text{ entrapment (\% w/w)}} \times 100$$

The results were reported as mean of two experiments and for each experiment the mean of three sample analysis were determined.

Particle size study

The vials containing the *in situ* formed PLGA microspheres were subjected to vacuum filtering, followed by washing and drying of the microspheres as before. The particle size of these isolated microspheres were then compared to the actual isolated microspheres using a Master Sizer X from Malvern Instruments, Inc. (Southborough, MA). The microspheres were first suspended in 100 ml of distilled water (containing 1% Tween 80) and subjected to

sonication for 30 sec and vortex mixing for 10 sec before analysis. The volume average particle size of three scans for each sample was reported.

RESULTS AND DISCUSSION

Formation of the various injectable biodegradable PLGA devices

The novel microencapsulation process on modification formed *in situ* implant or microspheres or isolated microspheres (Figs. 1). The mechanism of *in situ* implant formation was the same as described by others (Shah et al., 1993; Dunn et. al., 1995a,b; Frank et. al., 1994; Dunn et. al., 1994; Lowe et. al., 1993; Dunn et. al., 1992; Tipton et. al., 1992; Dunn et. al., 1991). A parenterally administered liquid on coming in contact with water from aqueous buffer or physiological fluid, solidifies into a gel matrix (implant) *in situ*, entrapping cytochrome *c* (Fig. 1). PLGA is a hydrophobic polymer and it precipitates in presence of water, thereby causing the matrix to harden. The optical micrograph of the *in situ* formed implant confirms its gel matrix structure (Fig. 2a).

We have previously described the *in situ* formation of microspheres which was based on the principle of coacervation (Jain et. al. a,b) (Figs. 1 and 2). This method formed PLGA microglobules which on coming in contact with water, hardened to form discreet PLGA microspheres (*in situ* formed microspheres) (Fig. 1). Cytochrome *c* was then released from these microspheres in a controlled fashion (Jain et. al. a,b). The optical micrograph of the *in situ* formed microspheres are shown in Fig. 2b.

The PLGA microglobule dispersion on injection into an aqueous PVA solution (with constant stirring) lead to the loss in integrity of the dispersion and rapid hardening of the PLGA microglobules into microspheres (Fig. 2). These were then isolated by washing and drying (Fig. 1). These microspheres exhibit a dense and rigid matrix structure and are smaller than the *in situ* formed microspheres (Fig. 2c).

Percentage cytochrome *c* encapsulation efficiency and particle size studies

As depicted in Table I, the *in situ* formed implant exhibits the lowest percentage cytochrome *c* encapsulation efficiency. There is a lag period between the injection of the liquid implant system and the actual formation of the implant. During this period, a significant amount of the drug is released into the aqueous buffer, leading to poor encapsulation efficiency (Table I). The PLGA microglobules when injected into the aqueous PVA solution (while stirring), cause rapid extraction of the vehicles and subsequent faster hardening of the microglobules into microspheres. Thus, not much drug is lost and the isolated microspheres exhibit an excellent encapsulation efficiency (Table I). For the *in situ* formed microspheres, the extraction of the vehicle mixture is gradual. There is transportation of the cytochrome *c* to the external aqueous buffer during the hardening of the PLGA microglobules, leading to some loss of cytochrome *c* (Table I). The isolated PLGA microspheres are produced relatively faster than the *in situ* formed microspheres and hence they are smaller in size as compared to the *in situ* formed microspheres (Table I).

In vitro cytochrome *c* release study

A strong burst (amount of cytochrome *c* released by the end of day 1) was observed for the *in situ* formed implant (Figure 3). This is chiefly due to the high concentration of unencapsulated cytochrome *c* as a result of low percentage encapsulation efficiency (Table I). The burst could also be due to dissolution of cytochrome *c* attached to the implant surface. The *in situ* formed implant exhibited the fastest release of cytochrome *c* (Figure 3). The isolated microspheres exhibited the lowest burst and this is due to the absence of the unencapsulated cytochrome *c* (Figure 3). Also they showed the slowest drug release (Figure 3). This could be attributed to the rigid and dense nature of their matrix which permitted slow release of cytochrome *c*. The *in situ* formed PLGA microspheres have a relatively low percentage encapsulation efficiency than the isolated microspheres (Table I) and hence they exhibit relatively higher burst effect. Also they are relatively more porous than the isolated microspheres,

therby exhibiting a relatively faster cytochrome *c* release than the isolated microspheres (Figure 3).

CONCLUSION

Controlled delivery of cytochrome *c* from the various biodegradable PLGA devices prepared by a novel, modified microencapsulation process is described here. The method produced different syringeable mixtures, which on injection formed solid matrix type implant or microspheres (*in situ* formed implant or microspheres respectively) entrapping cytochrome *c*. The process can also be made to yield injectable, isolated microspheres. The burst effect from these devices exhibited the following trend: *in situ* formed implant > *in situ* formed microspheres > isolated microspheres. The *in situ* formed microspheres were larger in size than the isolated microspheres. Also, the isolated microspheres exhibited the slowest controlled release of cytochrome *c*, while the *in situ* formed implant exhibited the fastest. Thus, this novel, modified microencapsulation process is versatile and it can produce various drug loaded injectable biodegradable PLGA devices having different characteristics.

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Figure 1-Schematic representation of the novel modified microencapsulation process to produce various injectable biodegradable PLGA devices

Figure 2a-Optical micrograph of the *in situ* formed PLGA implant

Figure 2b-Optical micrograph of the *in situ* formed microspheres

Figure 2c-Optical micrograph of the isolated microspheres

Table I-Percentage cytochrome *c* encapsulation efficiency and particle size studies of the various injectable biodegradable PLGA devices

Injectable biodegradable PLGA device	Percentage cytochrome <i>c</i> encapsulation efficiency	Particle size (μm)
<i>In situ</i> formed implant	52.8	—
<i>In situ</i> formed microspheres	74.5	98.6
Isolated microspheres	90.3	84.3

Figure 3-*In vitro* cytochrome *c* release from various injectable biodegradable PLGA devices

SECTION III

CONCLUSION AND FINAL REMARKS

The salient features of the novel microencapsulation process and the drug delivery system described in this research project are as follows:

- (1) The system excluded the use of unacceptable organic solvents like methylene chloride and used acceptable vehicle mixture instead to prepare biodegradable PLGA microspheres.
- (2) The system formed drug containing PLGA microglobules ("premicrospheres" or "embryonic microspheres") which could be considered as precursors to the final microsphere product; these on coming in contact with water hardened to form discrete PLGA microspheres (*in situ* formed microspheres) which subsequently exhibited non-variable, predictable, and controlled drug release profile.
- (3) Unlike the traditional methods, this system precluded the need for reconstitution of the PLGA microspheres as they are formed *in situ*.
- (4) Various formulation variables affected the characteristics of this system.
- (5) The formulation and process conditions did not adversely affect the physical stability of the encapsulated protein drugs.
- (6) Besides *in situ* forming microspheres, the novel microencapsulation method can be modified to produce *in situ* formed implant or isolated microspheres; these drug loaded devices exhibited different characteristics.
- (7) This research project makes a significant overall contribution to the knowledge of the underlying theoretical principles of drug delivery

through biodegradable devices and in particular, problems associated with protein drug delivery.

- (8) The novel nature of the system provides a high probability that a patent application would be filed.

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