FABRICATION AND CHARACTERIZATION OF CONTROLLED RELEASE OF PROTEINS AND PEPTIDES FROM POLY GLU LACTIDE-CO-GLYCOLIDE (PLGA) MICROSPHERES

BY

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ABSTRACT

Biodegradable poly(lactide-co-glycolide) (PLGA)-based microspheres and nanoparticles have received much attention over the last twenty-five years for controlled parenteral delivery of therapeutic protein and peptide drugs. In general, PLGA-based injectable delivery systems of macromolecular protein and peptide drugs still suffer from two major technical problems associated with their inherent stability problem. Initial burst release followed by very slow and incomplete release is one of the most serious problems in the formulation of PLGA-based protein drugs delivery system. In this study, two model proteins, bovine serum albumin (BSA) and lysozyme, and a therapeutic peptide drug, insulin loaded double-walled microspheres have been fabricated using a fast degrading glucose core, hydroxyl-terminated poly(lactide-co-glycolide) (Glu-PLGA) and a moderate degrading carboxyl-terminated PLGA polymers to reduce the high initial burst release and to eliminate the lag phase from the release profile of PLGA microspheres. Double-walled microspheres were prepared using a modified water-in-oil-in-oil-in-water ($w_1/o/o/w_2$) method. In addition, single-polymer microspheres were prepared by a conventional water-in-oil-in-water ($w_1/o/w_2$) emulsion solvent evaporation method for comparison. The microspheres size, morphology, encapsulation efficiency, thermal properties, *in vitro* drug release, and structural integrity of BSA, lysozyme and insulin were evaluated in this study. The bioactivity of released lysozyme was determined using *Micrococcus lysodeikticus* as substrate. Moreover, *in vivo* release and bioactivity of insulin was evaluated upon subcutaneous injection of insulin loaded microspheres in STZ induced diabetic rats. BSA, lysozyme and insulin loaded double-walled microspheres prepared with Glu-PLGA and PLGA polymers in a mass ratio of 1:1 showed reduced particle size (< 5 μm), non-porous, smooth-surfaced, and spherical in shape. In contrast, highly porous surface was observed for single-polymer microspheres. Double-walled microspheres comprising Glu-PLGA and PLGA polymers in a mass ratio of 1:1 exhibited higher encapsulation efficiency for BSA compared to lysozyme and insulin. A significant reduction in initial burst release was achieved for double-walled microspheres compared to single-polymer microspheres. In addition, double-walled microspheres prepared using Glu-PLGA and PLGA polymers in a mass ratio of 1:1 exhibited continuous and almost complete release of BSA and insulin after small initial burst release without any lag phase. In contrast, incomplete release was observed for lysozyme from both double-walled and single-polymer microspheres. SDS-PAGE result shows that a small fraction of encapsulated and released proteins (BSA and lysozyme) underwent aggregation and possible degradation, whereas no substantial aggregation or degradation was observed for insulin during microspheres fabrication and *in vitro* release studies. Moreover, the *in vivo* studies demonstrated that the bioactivity of insulin was retained throughout the experimental period. This study suggests that double-walled microspheres made of Glu-PLGA and PLGA polymers in a mass ratio of 1:1 can be a potential delivery system for pharmaceutical proteins and peptides.

خلاصة البحث

حاز البوليمير المنخرب حيوياً بولي لاكتيد-كو-غليكوليد (PLGA) والمكروسفير والنانوسفير المحضرة منه اهتماماً كبيراً في الخمس وعشرين سنة الماضية للإيتاء الحقني المديد للبروتينات والببتيدات العلاجية. بشكل عام لا تزال أنظمة الإيتاء الحقنية للبروتينات والببتيدات المعتمدة على الـPLGA تعاني من مشكلتين تقنيتين أساسيتين مرتبطتين بالثباتية. التحرر الفجائي المبدئي المتبوع بالتحرر البطيء وغير الكامل هو حقاً مشكلة أساسية. في هذا البحث تمت كبسلة بروتينين نموذجيين هما الألبومين السيرومي البقري (BSA) واللليزوزيم والببتيد العلاجي الإنسولين في مكروسفيرات ثنائية الجدار باستخدام البوليمير سريع التخرب ذو النواة الغلوكوزية (Glu-PLGA) وبوليمير PLGA آخر متوسط التخرب ذو نهاية طرفية كربوكسيلية ، من أجل إنقاص التحرر الفجائي المبدئي المرتفع وأيضاً لإزالة زمن التأخير من نموذج التحرر من المكروكبسولات. تم تصنيع المكروسفيرات ثنائية الجدار بطريقة معدلة من طريقة المستحلب ماء/زيت/زيت/ماء. إضافة إلى ذلك، تم تصنيع المكروسفيرات أحادية البوليمير باستخدام الطريقة التقليدية مستحلب ماء/زيت/ماء المتبوعة بتبخير المحل. تم تقييم المكروسفيرات من ناحية الأبعاد، فعالية الكبسلة، الخواص الحرارية، تحرر الدواء في الزجاج، وتكامل بنية للبروتينات. تم تقييم الفعالية الحيوية لليزوزيم باستخدام *Micrococcus lysodeikticus*. إضافة إلى ذلك تم تقييم التحرر في الحي والفعالية الحيوية للإنسولين بعد الحقن تحت الجلد للمكروسفيرات المحملة بالإنسولين في الجرذان ذات الداء السكري المحرض بالـSTZ. المكروسفيرات المحملة بالبروتينات وذات الجدار المضاعف المحضرة من البوليميرين Glu-PLGA وPLGA بنسبة 1:1 أظهرت نقصاناً في الأبعاد (أقل من 5 مكرون)، غير مسامية، ملساء السطح، وكروية الشكل. بالمقابل، أظهرت المكروسفيرات وحيدة البوليمير مسامية سطحية عالية. المكروسفيرات ثنائية الجدار المكونة من البوليميرين Glu-PLGA وPLGA بنسبة كتلة 1:1 أظهرت فعالية كبسلة أعلى للبروتين BSA مقارنة باللليزوزيم والإنسولين. تم تحقيق نقصان مهم في التحرر الفجائي المبدئي باستخدام المكروسفيرات ثنائية الجدار مقارنة بالمكروسفيرات وحيدة البوليمير. إضافة إلى ذلك، المكروسفيرات المحملة بالبروتينات وذات الجدار المضاعف المحضرة من البوليميرين Glu-PLGA وPLGA بنسبة 1:1 أظهرت أيضاً تحررا مستمراً وشبه كامل للـBSA والإنسولين بعد تحرر فجائي مبدئي صغير ومن دون زمن تأخير. بالمقارنة، تمت ملاحظة تحرر غير كامل لليزوزيم من كلا المكروسفيرات ثانئية الجدار وأحادية البوليمير. نتائج فحص الـSDS-PAGE أظهرت أن جزءاً صغيراً من البروتينات المكبسلة والمتحررة (BSA واللليزوزيم) تعرضت للتكتل ومن المحتمل التخرب، بينما لم تتم ملاحظة تكتل أو تخرب جوهري للإنسولين أثناء تحضير المكروسفيرات وخلال فترة التحرر في الزجاج. أضف إلى لك، بينت الدراسة في الحي أن تكامل البنية والفعالية للإنسولين قد تم الحفاظ عليها على طول فترة التجارب. كخلاصة، تقترح هذه الدراسة أن الكروسفيرات ثنائية الجدار الحضرة من البوليميرين Glu-PLGA وPLGA بنسبة 1:1 يمكن أن تمثل نظام إيتاء محتمل للبروتينات والببتيدات الصيدلانية.

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APPROVAL PAGE

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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LIST OF ABBREVIATIONS

AOT	Docusate sodium salt
BSA	Bovine serum albumin
BF	Bovine serum albumin formulation
CyA	Cyclosporin A
DCM	Dichloromethane
DMAB	Didodecyl dimethyl ammonium bromide
DMSO	Dimethyl sulfoxide
DSC	Differential scanning calorimetry
EA	Ethyl acetate
EE	Encapsulation efficiency
EVA	Poly(ethylene-co-vinyl acetate)
FITC-BSA	Fluorescein isothiocyanate labeled bovine serum albumin
Glu-PLGA	Glucose core poly(lactide-co-glycolide)
HSA	Human serum albumin
IBU	Ibuprofen
IF	Insulin formulation
LC	Loading capacity
LF	Lysozyme formulation
MCA	Metoclopramide HCl
MPa	Mega-Pascals
PBS	Phosphate buffer saline solution
PCL	Polycaprolactone
PELA	Poly(D, L-lactide)-poly(ethylene glycol)
PGA	Polyglycolide
PLA	Polylactide
PLGA	Poly(D, L-lactide-co-glycolide)
PLHMGA	Poly(lactic-co-hydroxymethyl glycolic acid)
POE	Poly(orthoester)
PPF	Precision particle fabrication
PS	Polystyrene
PVA	Poly(vinyl alcohol)
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
STZ	Streptozotocin
T*g*	Glass transition temperature
THF	Tetrahydrofuran

CHAPTER ONE

INTRODUCTION

1.1 POLYMERIC MICROSPHERES AND NANOPARTICLES AS DRUG DELIVERY SYSTEMS

Biodegradable polymeric microspheres and nanoparticles are solid or semisolid colloidal spherical particles in which the drug substance is either dispersed or dissolved depending on its solubility. Polymeric particles are classified based on their size; the diameter of microspheres ranging from 1 to 250 μm while nanoparticles having size ranging from 10 nm to 1000 nm (Soppimath et al., 2001). Micro and macromolecular drugs are both encapsulated in polymeric particulate devices and can be used as drug carriers. The drug can be adsorbed, dissolved, entrapped, or encapsulated into the nanoparticles and microspheres matrix.

Nanoparticles, nanocapsules, microspheres, and microcapsules can be obtained with the same polymer depending on the methods of preparation (Barratt, 2000; Letchford and Burt, 2007). Nanoparticles and microspheres are matrix systems in which drug particles are uniformly dispersed. On the other hand, nanocapsules and microcapsules are reservoir systems consisting of a drug-containing core surrounded by a rate controlling biodegradable polymer shell. After preparation, the drug loaded micro and nanoparticles are usually dispersed in aqueous solution before administration. The prepared drug-loaded micro or nanoparticles can be administered to humans by a number of routes such as parenteral route, oral route, or applied topically either to the eye or the skin. Moreover, nanoparticles can be used for pulmonary delivery by inhalation. The conventional drug delivery system does not usually provide rate-controlled release or target specific release. It has been observed

that conventional drug administration system provides a very sharp increase of drug concentration at potentially toxic levels, which follows a relatively short period at the therapeutic level and drug concentration eventually decreases until re-administration. On the other hand, particulate drug delivery systems (i.e. microspheres and nanoparticles) are used as drug carriers to deliver drugs in the areas of interest and to release the encapsulated drug slowly over a desired period of time by which the effective local drug concentration is maintained. Due to the advantages of sustained release and targeted delivery, biodegradable polymeric micro and nanoparticles have been investigated extensively for the last two decades.

Biodegradable polymeric microspheres/nanoparticles exhibit three major advantages over conventional drug administration. First, polymeric microspheres or nanoparticles demonstrate a higher surface area to volume ratio compared to conventional drug carriers due to their micro or nano scale size. The high surface area changes particle surface properties and the interactions with disperse phase, especially with respect to the dissolution rate (Merisko and Liversidge, 2008). More than 40% of the therapeutic compounds are poorly water soluble, and their clinical usefulness are greatly limited by their bioavailability (Rasenack and Müller, 2002; Elgart et al., 2012). Formulating these hydrophobic drugs into a micro or nanoparticle form could efficiently improve their dissolution rates, and eventually improve their performance. Second, sustained release of a therapeutic agent could be achieved by encapsulating it within different polymers. A number of polymers have been investigated to encapsulate drug compounds including naturally occurring such as chitosan and serum and synthetic polymers such as polylactide (PLA), polyglycolide (PGA), poly(D,L-lactide-co-glycolide) (PLGA) and polycaprolactone (PCL). Sustained or triggered release of drug from micro and nanoparticles could be achieved by selecting

polymers, process parameters and methods of preparation (Cai et al., 2009). Third, targeted delivery of an encapsulated drug could be achieved by coupling it to a molecule with an affinity for a particular target such as cancer cells (Chittasupho et al., 2009). This targeted drug delivery system (DDS) has tremendous significance for the highly toxic or carcinogenic drugs which are commonly prescribed for the treatment of cancer. The majority of anti-cancer drugs are considered as highly biotoxic to most kinds of cells, both cancerous and healthy. Targeted delivery of these anti-cancer drugs protects normal tissue and thus would minimize the destructive side effects of chemotherapy.

1.2 PLGA-BASED MICROSPHERES AND NANOPARTICLES FOR PROTEIN/PEPTIDE DRUGS DELIVERY

Insulin, a potent molecule for treatment of diabetes, was discovered by Banting and Best in 1921. Since then, extensive research has been going on to explore the most effective and convenient route of its administration (Brown, 2005). In addition to subcutaneous injection, various non-invasive routes such as oral, rectal, vaginal, buccal, pulmonary and nasal routes have been examined, but so far researchers have not yet developed a successful formulation for its clinical application (Sheshala et al., 2009; Rekha and Sharma, 2013). In response to the growing advances in biotechnology and chemistry, the number of recombinant proteins and peptides available for therapeutic purposes is increasing significantly (Andersen, 2002; Wurm, 2004). Proteins are macromolecules, consisting of one or more long chain amino acid residues. Amino acid chains with less than 40 residues are usually referred to as peptides. Unlike low molecular weight drugs, proteins and peptides have different structures such as primary, secondary, tertiary and in some cases, quaternary structure

with labile bonds and side chains with chemically reactive groups. The primary structure provides the sequence of different amino acids held together by covalent peptide bonds. The secondary structure refers to highly regular local sub-structures. There are two main types of secondary structure; α-helix and β-sheet. The tertiary structure presents a three-dimensional structure of a single protein molecule. The α-helix and β-sheets are folded into a compact globule. This is driven by a number of non-covalent interactions and hydrophobic packing i.e. the affinity to the burial of hydrophobic residues from water. However, specific interactions such as salt bridges and disulfide bonds are necessary to stabilize the three dimensional structure. The quaternary structure is a larger assembly of several protein molecules. Many proteins do not have the quaternary structure and are active as monomers. However, expectations concerning the delivery of peptide and protein-based therapeutics for the treatment of chronic and life threatening diseases have been limited due to their short biological half-life, fragile structure and low oral bioavailability (Goddard, 1991; Yun et al., 2013). In addition, most of the protein therapeutics is available in the market in an injectable form and patients that require chronic treatment with such therapeutics often require repetitive injections to achieve the desired therapeutic effects resulting in a low patient compliance (Brown, 2005). Therefore, there is a need of a delivery system which can release these biologically active molecules continuously for days to months.

Substantial research efforts have been focused to protect therapeutic proteins/peptides from proteolysis and to obtain a controlled release and improved pharmacokinetic profiles after injection (Perez et al., 2002). The most investigated technique is to encapsulate therapeutic protein/peptide drugs in biodegradable polymeric microspheres and nanoparticles that release the drug slowly and

continuously due to the gradual degradation of the polymer matrix after hydration and also cleavage of sensitive bonds present in the polymer chain. Various therapeutic peptides and proteins encapsulated PLGA micro and nanoparticles have received much attention over the last twenty-five years for their sustained release application over an extended period (Kim and Park, 2004; Srinivasan et al., 2005; Geng et al., 2008; Samadi et al., 2013). Since this technology provides unique advantages over traditional delivery approaches (e.g. improved drug efficacy and patient compliance), several formulations of proteins based on biodegradable micro/nanoparticles have already been marketed, as shown in Table 1.1 (Sinha and Trehan, 2003; Misra, 2010).

In general, PLGA-based injectable delivery systems of macromolecular protein and peptide drugs still suffer from three major technical problems associated with their inherent stability problem. Initial burst release followed by very slow and incomplete release is three most serious problems in the formulation of PLGA-based protein drug delivery system. Initial burst release means a rapid release of a large portion of the encapsulated drug during the first few hours of incubation (Huang and Brazel, 2001). It occurs due to the immediate dissolution of the surface-bound drug as well as the rapid diffusion of hydrophilic protein and peptide drugs through the pre-existing pores and channels present in the microspheres matrix (Wang et al., 2002a; Manoharan and Singh, 2009).

Table 1.1 Marketed Formulations of Proteins Based on Biodegradable Microspheres

Polymer	Drug	Trade Name	Company	Route of Administration	Application
PLGA	Leuprolide acetate	Lupron Depot®	Takeda-Abott	3 months depot suspension	Prostate cancer
PLGA	Recombinant human growth hormone	Nutropine Depot®	Genentech-Alkermes	Monthly S/C injection	Growth hormone deficiency
PLGA	Goserelin acetate	Zoladex®	Astra Zeneca	S/C implant	Prostate cancer
PLGA	Octreotide acetate	Sandostatin LAR® Depot	Novartis	Injectable S/C suspension	GH suppression, anticancer
PLGA	Triptorelin	Decapeptyl®	Debiopharm	Injectable depot	Prostate cancer
PLGA	Lanreotide	Somatuline® LA	Ipsen	Injectable depot	Acromegaly
PLGA	Buserelin acetate	Suprecur® MP	Aventis	S/C implant	Prostate cancer

It has been reported that about 10 to 80% of the loaded drug is released within a very short period (Ahmed et al., 2012). The initial burst release poses a serious toxicity threat as excessive release rates could result in drug levels that are close to or exceed toxic threshold levels. Moreover, microspheres tend to have a very slow release of the drug (near to zero) after the initial burst. The slow or no release period is termed as the “lag phase” or “induction period” which continues until the extensive degradation of PLGA starts. During the induction period, the patients may not be treated effectively due to release of insufficient drug (Wang et al., 2002a). In some studies, a significant fraction of the loaded protein was not released after the bulk degradation of the polymer, either due to protein aggregation or adsorption to the strong hydrophobic surface of the polymer, resulting in an incomplete protein release (Bittner et al., 1998; van de Weert et al., 2000a; Jiang et al., 2002a; Kim and Park, 2004). Moreover, due to the accumulation of PLGA degradation products (lactic acid