Preparation and Characterization of Poly (D,L-Lactide-Co-Glycolide) Microspheres for Controlled Release of KSL Peptide

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Abstract :

The purpose of this research was to prepare, characterize, and evaluate the new antimicrobial peptide KSL peptide encapsulated in poly(D,L-lactide-co-glycolide) (PLGA)composite microspheres. KSL was loaded in poly(acryloyl hydroxyethyl) starch (acHES) micropar-ticles, and then the peptide-containing microparticles were encapsulated in the PLGA matrix by a solvent extraction /evaporation method.

KSL-loaded PLGA microspheres were also prepared without the starch hydrogel microparticle microspheres for comparison study. KSL peptide microspheres were characterized for drug content, surface morphology, microspheres size determination, polymers stability , in vitro microspheres degradation and in vitro release. KSL peptide encapsulation efficiency resulted in about 98% for RG503 microspheres and AcHES- RG503 composite microspheres. Microspheres mean diameters were 11.12µm and 28µm for RG503 microspheres and AcHES- -RG503 composite microspheres respectively. Differential scanning calorimetry (DSC) analysis showed no structural changes in the polymers after KSL peptide loading. The morphological effects and polymers degradation were analyzed to obtain a better understanding of the mechanism of KSL peptide release from microspheres and composite microspheres. Microspheres incubated in 0.1M phosphate buffer saline, pH 7.4 at 37°C were hydrated and started to degrade as shown by gel permeation chromatography (GPC) analysis. The result indicated that the release of KSL peptide from microspheres was due to the bulk degradation. In vitro release profile showed that the microspheres type significantly affect the release of KSL peptide. In vitro KSL peptide release after 60 days incubation in 0.1M phosphate buffer saline, pH 7.4 at 37°C were 82.23% and 62.12% from 10% KSL peptide loaded AcHES-RG503 composite microspheres and 10%KSL peptide loaded RG503 microspheres respectively.

Key words :KSL peptide , microspheres, composite microspheres, PLGA

الخلاصة

الغرض من هذا البحثِ هو تحضير وتشخيص وتقييم ببتيد ال KSL المضاد ألجرثومي الجديد في كرات مجهريه معوضه متكونة من متعدّدِ حامض اللبن وحامض الكلايكولك . حُمّلَ ببتايد ال KSL في متعدّدِ جبيبات (acryloyl hydroxyethyl) النشا ، وبعد ذلك غلفت هذه الحبيبات المحتوية على peptide ال KSL في مصفوفة متعدّدِ حامض اللبن وحامض الكلايكولك (PLGA) بطريقة استخلاص المذيب / التبخير. حضرت كرات ال PLGA المجهرية المحملة ببتيد ال KSL خالية من حبيبات ال hydrogel النشوية لعمل دراسة مقارنة. تم تعريف محتوى الدواء للكرات المجهرية لببتيد ال KSL وكذلك ، الشكل الخارجي , الحجم، إستقرارية البوليمرات ,تجزئة الكرات المجهرية خارج الجسم والتحرر خارج الجسم. ان فعالية تحميلببتيد ال KSL كانت بحدود 98 % الى كرات RG503 المجهرية والى كرات AcHES-RG503 المجهرية المعوضه. لقد كان معدل قطر الكرات المجهرية كان 11.12مايكرون و 28 مايكرون للكرات المجهريةRG503 و الكرات المجهرية المعوضةAcHES-RG503 على التوالي . تحليل المسح التفريقي للسعرات (Differential Scanning Calorimetry) اظهر عدم وجود تغيير في تركيب البوليمرات بعد تحميل ببتيد ال KSL. التأثيرات على المظهر الخارجي وتجزئة البوليمرات قيمت للحُصُول على فَهْم أفضل لآليةِ تحرر peptide ال KSLمِنْ الكرات المجهرية والكرات المجهرية المعوضة. حضنت الكرات المجهرية في دارئ الفوسفات ذو الدالة الحامضية7.4 والتركيز المولاري 0.1 وبدرجة حرارة 37 مئوية ادى الى ترطيبها وتجزئها كما ظهر جليا من خلال التحليل الكروماتوكرفي الهلامي التنافذي (Gel permeation Chromatography) . وقد أشارت النتائج على إن تحرر ببتايد ال KSL كان من خلال ميكانيكية التكسر الكتلي. إن التحرر خارج الجسم الحي اشار الى إن نوع الكرات المجهرية يؤثر بشكل كبير على تحرر ببتايد ال KSL . لقد كان تحرر ببتيد ال KSL بعد 60 يوما من الحضن في دارئ الفوسفات ذو الدالة الحامضية 7.4 وتركيزه المولاري 0.1 وبدرجة 37 درجة مئوية هو بنسبة 82.23% و62.12% من الكرات المجهرية المحملة ببتايد ال KSL بنسبة 10% التالية RG503 وAcHES-RG503 على التوالي.

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Introduction :-

Recently , a large number of recombinant proteins and synthetic peptides have been developed as potential therapeutic agents[1]. Poor absorption of these agents by the oral route due to their poor physicochemical properties or due to a high first-pass metabolism in the liver or degradation in the acidic atmosphere of the stomach. The digestive enzymes in the intestine or in the gut wall are responsible for the presystemic degradation of many drugs [2].These obstacles has necessitated delivery by alternative routes, principally the parenteral route. Injectable controlled-release formulations are designed to release drugs in a controlled, predetermined fashion and have been developed in an effort to provide more consistent pharmacodynamic effects and minimize adverse effects (characteristics that are particularly useful for drugs with a narrow therapeutic index). Most commonly, active agents are incorporated within a biodegradable and bio-compatible polymeric matrix[3]. Biodegradable polymeric matrix has been found promising for delivering proteins and peptides over a desired period of time. Nowadays many polymer matrix materials are available, they gradually degrade after the drug is released at the desired site in the body [4-6]. Furthermore, administration of the drug by injection is possible if it is dispersed into microspheres [7-9] Microspheres are fine spherical particles containing active drugs. Microspheres have a diameter of less than approximately 1000 µm in which the drug is dissolved or dispersed homogeneously throughout the polymer matrix [3]. PLGA, an FDA-approved material, has been extensively studied for its biocompatibility, toxicology, and degradation kinetics[10]. PLGA is biocompatible, and more importantly, the degradation rates of PLGA and the accompanying release of encapsulated drugs can be controlled by the polymer's physicochemical properties such as molecular weight, hydrophilicity, and the ratio of lactide to glycolide [11]. Thus, it is possible to obtain the desired drug release from PLGA microspheres by altering the polymer's characteristics. Biodegradable polymeric microspheres have been shown to be effective in enhancing drug targeting specificity, lowering systemic drug toxicity, improving treatment absorption rates, and providing protection for pharmaceuticals against biochemical degradation [12]. Several peptides have been successfully incorporated into a PLGA matrix as a depot formulation for parenteral use[1].The drug release from biodegradable microspheres is governed by degradation rate of PLGA copolymer, which largely depends on the physical properties of polymer such as molecular weight, hydrophilicity, and the ratio of lactide to glycolide[13].proccing conditions employed during preparation of microspheres determine the properties of the microspheres , such as the size , morphology, encapsulation efficiency , and drug distribution and all these properties influence the release of drug from the delivery system[6]. A novel decapeptide, KKVVFKVKFK (**Lys^{1}-lys^{2}-Val^{3}-Val^{4}-Phe^{5}-Lys^{6}-Val^{7}-Lys^{8}-Phe^{9}-Lys^{10}**) (KSL peptide) has been identified that shows activity against C.albicans and a broad range of antibacterial activity but without hemolytic activity [14]. KSL peptide exhibited an ED99 (the dose at which 99% killing was observed after 15 min at 37℃) of 6.25 µg/ml against selected strains of Lactobacillus salivarius, Streptococcus mutans, Streptococcus gordonii and Actinobacillus actinomycetemcomitans. In addition, KSL peptide damaged bacterial cell membranes and caused 1.05 log units reduction of viability counts of saliva bacteria. In vitro toxicity studies showed that KSL peptide, at concentrations up to 1 mg/ml, did not induce cell death or compromise the membrane integrity of human gingival fibroblasts[15]. In previous study , the preparation and characterization of PLGA polymerRG502, RG502H and RG503H, microspheres and AcHES-RG503H composite microspheres loaded KSL peptide were investigated. The study showed that the in vitro KSL peptide release after 60 days incubation in 0.1M phosphate buffer saline, pH 7.4 37℃ were 75.13%, 52.27%,, 21.41%, and 30.69% from 10% KSL peptide loaded RG502, RG502H and RG503H, microspheres and AcHES-RG503H composite microspheres respectively [16].In this study , KSL loaded PLGA polymer RG503 and AcHES-RG503 composite microspheres were prepared for prolonged KSL release .

Materials and Methods :

Materials:

KSL decapeptide and Polyvinyl alcohol (PVA)(M.wt 3000-7000) were purchased from Sigma Chemical (St. Louis, MO). Poly (D, L-Lactic-co-glycolic acid) (PLGA) polymer with hydrophobic end group [Molecular weight (M.wt) 30 Kilodalton(KD) (KD= kilogram/mole)], copolymer ratio=50:50, Rosmer® RG503). Poly(Acryloyl Hydroxyethyl starch)(AcHES) was purchased from Dupont Pharmaceutics (Wilmington, DE, USA).All other chemicals reagents used were of analytical reagent grade.

Methods:

Preparation of Microspheres:

The KSL peptide microspheres were prepared with PLGA polymerRG503 (30KD) . The 10% loading of KSL peptide microspheres were prepared by solvent extraction/evaporation technique[17]. The preparation procedure was as follows: A methylene chloride (CH2Cl2) solution that contained approximately 25 % (w/w) of polymer was mixed with a methanolic solution (CH3OH) of KSL peptide. The resulting mixture [dispersed phase (DP)] was then slowly added to 50 ml of 0.35% (w/w) PVA aqueous solution [continuous phase (CP)] and maintained at 25 °C with a water bath. The mixture of CP and DP were emulsified for 5 min using a mixer. The temperature of the emulsion was increased and maintained at 40°C to extract and evaporate the organic phase over 1hr, then temperature of the system was reduced to 25 °C and microspheres were recovered on 5µm pore size filter paper and then freeze dried over night.

Preparation of KSL Peptide-Loaded AcHES-RG503 Microspheres:

The AcHES-RG503 composite microspheres were prepared by a modified solvent extraction/ evaporation method (18) with 10% (w/w) loading of KSL peptide. 50 mg KSL peptide was dissolved in 0.250 ml of distilled water. The solution was added to 50mg AcHES particles, and the particles were allowed to swell for 5 minutes with vortex mixing at room temperature. A 30% w/w RG503 methylene chloride solution was added to the swollen AcHES particles and vortex for 2 minutes at room temperature to form a dispersion of KSL peptide in hydrogel/ polymer in solvent. This primary dispersion was then added to precooled (4 °C) 100 ml 6.0% PVA solution and stirred by a silverson mixer at 2500 rpm for 2 minutes. The resulting secondary suspension was transferred to one liter distilled water and stirred gently for 2hr at room temperature to remove the organic solvent and solidify the polymer. The microspheres were filtered through 5µm filter paper. Then washed 3 times with 2 liters distilled water and freeze-dried over night.

Characterization of the Prepared Microspheres:

KSL Peptide Content and Loading Efficiency Determination of the Prepared Microspheres:

KSL peptide content of peptide loaded RG503 microspheres and AcHES-RG503 composite microspheres were determined by HPLC(Reverse-phase-performance liquid chromatography, Shimadzu Scientific instrument, Inc.Columbia,MD.) as follows: Triplicate samples of 10 mg of the microspheres were quantitatively transferred to a 12 ml glass test tube. The matrix was solubilized in 2 ml of methylene chloride , then 10 ml of 0.1M acetate buffer , pH 4.0 was added and the test tubes were agitated by a wrist action shaker for 1hr. Samples were centrifuged at 3000 rpm and the aqueous layer was analyzed by HPLC. A second extraction with 10 ml of acetate buffer was made to ensure complete extraction and effect mass balance. The KSL peptide concentration was determined by HPLC (Shimadzu Scientific instrument, Inc.Columbia,MD.)[15] using a prosphere C-18 column (4.6x250mm,Altech, Deerfield,,IL). Gradiant elution was accomplished with 0.1% trifluoroacetic acid in water(phase A) and 0.1% trifluoroacetic acid with acetonitrile (phase B) with increasing the amount of phase B from 20% to 30% over 10 minutes at a flow rate of 1.2ml/min.The running time was 20 minutes and the U.V. detection was at 215nm. The injection volume was 40µl. The KSL amount was determined using calibration curve according to

(y= -1.72542.4521 +13927.4152x , r=0.997)

The loading efficiency was calculated using the following equation.

$$\text{Loading efficiency (\%)} = \frac{M\ (\text{actual})}{M\ (\text{theoretical})} \times 100$$

Where M actual is the actual amount of KSL peptide in microspheres and M theoretical is the amount of KSL peptide in microspheres calculated from the quantity added in the fabrication process[19].

Morphology Study of the Prepared Microspheres:

A scanning electron microscope(Hitachi Model 5800, Japan.) was used to exam the shape and surface morphology of the microspheres .freeze- dried microspheres were mounted on adehisve stub and then coated with gold palladium under vacuum. The coated specimen was then examined under the microscope and photographed.

Microsphere Size Determination:

The size of KSL peptide loaded RG503 microspheres and KSL peptide loaded AcHES-RG503 composite microspheres were analyzed by using laser diffraction(Malvern instrument , Malvern, UK.).

The microspheres were suspended in 0.1% aqueous tween80 solution and a 100-mm(for size range of 1.9-188µm) focal length was employed to determine particle size, while the sample was stirred at 100 rpm in the sample cell using a magnetic stirrer bar. All particles size measurements were repeated three times per sample and each sample was prepared in

triplicate. The average particle size was expressed as the volume mean diameter VMD in microns.

Differential Scanning Calorimetry (DSC) Analysis:

The changing in the structure of the PLGA polymers during the loading by KSL peptide can be predicted by measuring the glass transition temperature (Tg) of PLGAs before and after loading. The effect of loading on the Tg of PLGA polymers was investigated by the DSC(Mettler TA 2920, AC USA.). Samples of the prepared microspheres, about 5mg of blank and KSL peptide loaded RG503 and AcHES-RG503 composite microspheres were scanned at 5°C/min. heating rate in the range of 0-80°C.

The Molecular Weight Degradation Study of the PLGA Biodegradable Polymers Using Gel Permeation Chromatography (GPC):

The molecular weight distribution of blank and KSL peptide loaded RG503 microspheres and KSL peptide loaded AcHES-RG503 composite microspheres incubated in PBS pH 7.4 at 37°C were determined by gel permeation chromatography[A waters M-45 solvent delivery system with a Shimadzu SPD 6AV UV-Vis Spectrophotomereic Detector (ë=220nm)]. Two Ultrastyragel columns connected in series (7.8x300 mm each, one 10^4Á pores and one with 10^3Á pores) were used. Samples, 5 mg/ml, were eluted with tetrahydrofuran at 0.4 ml/min. The average molecular weight of each sample was calculated using monodisperse polystyrene standards, molecular weight 1000-90000 Dalton.

In Vitro KSL Peptide Release Study from the Prepared Microspheres:

Fifty mg of KSL peptide loaded RG503 microspheres and 10% KSL peptide AcHES-RG503 composite microspheres were incubated with 40 ml of 0.1 M PBS, pH 7.4 containing 0.02 % sod. azide as preservative to prevent the growth of microorganisms during the incubation at 37°C in a temperature - controlled oven. Separate test tubes with equal amounts of microspheres were maintained for each time point of the release study. The sampling time were 2, 5, 10 , 20, 30, 40, and 60 days. Test tubes were shaken twice weekly to simulate the static in vivo condition and the peptide release was determined from the residual microspheres . At each time point, remaining microspheres were recovered by centrifugation and assayed for drug content. The supernatant was removed and assayed for the KSL peptide amount to be used in the calculation of mass balance study. All analysis was carried out by HPLC on triplicate samples.

Results and Discussion :

KSL Peptide Content and loading Efficiency of the Prepared Microspheres:

The amount of KSL peptide encapsulated in each of the polymeric microspheres and composite microspheres was determined by calculating the amount of KSL peptide after dissolving microspheres and composite microspheres in of methylene chloride and extracting the KSL peptide in acetate buffer. The results indicated that about 98% of the total peptide could be recovered as shown in table (1).The loading efficiency of each batch was similar and independent of the biodegradable polymers used from the preparation of microspheres. The same high percentage of peptides encapsulation were obtained by researchers when they used CH3OH and CH2Cl2 system as the organic phase of O/W emulsion [20].

Table 1: Batch Summary Of KSL Peptide Loading Microspheres.

Microsp-heres batch	Theoreti cal weight. of KSL peptide (mg)	Actual weight KSL peptide loading (mg)	KSL peptide loading efficiency (%)
RG503	**10**	**9.82**	**98.2**
AcHES-RG503	**10**	**9.84**	**98.4**

Morphology of the Prepared Microspheres:

Scanning electron microscopy(SEM) images of the 10% KSL peptide loaded RG503 microspheres and 10%KSL peptide loaded AcHES-RG503 composite microspheres after preparation indicated the formation of nonporous spheres with relatively smooth surfaces(figures 1and 2). The similar morphology may be due to the same experimental conditions.

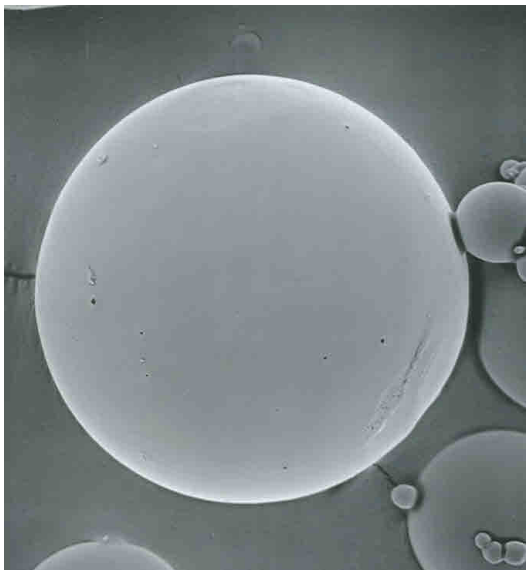

Figure 1: Scanning electron microscopy of KSL peptide loaded RG503 microspheres

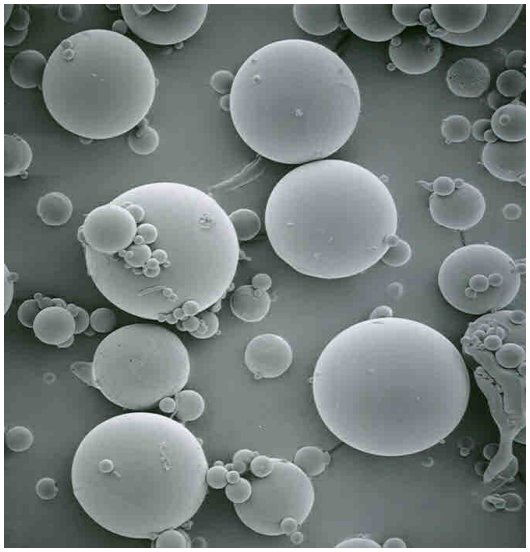

Figure 2: Scanning electron microscopy of KSL peptide loaded AcHES- RG503 microspheres.

Microsphere Size:

The mean microsphere size were at 11.12 and 28µm for RG503 microspheres and AcHES-RG503 composite microspheres respectively,. that are suitable for intramuscular or subcutaneous administration. The smaller RG503 microspheres size, compared to the AcHES-RG503 composite microspheres could be due to lower viscosity of polymer solution of the RG503[17].

The Differential Scanning Calorimetry (DSC) Analysis of the Prepared Microspheres:

The glass transition temperature (Tg) of RG503 microspheres and AcHES-RG503 composite microspheres batches were determined by the DSC for blank and loading microspheres. No significant differences in Tg between KSL peptide loaded microspheres and the blank one (as shown in table (2)), which suggest that the KSL peptide loading has no effect on the internal structure of the polymers.

Microspheres	Tg (°C)
Blank RG503	54.15
KSL peptide loaded RG502	52.65
Blank AcHES- RG503H	53.58
KSL peptide loaded AcHES-RG503H	52.36

Table 2: The Glass Transition Temperature (Tg) Of Blank An Loaded Microspheres.

Degradation Result of the Prepared Microspheres :

Table (3) and figure (3) show that the erosion of KSL peptide-loaded PLGA microspheres leads to molecular weight-loss profiles that are in agreement with the kinetics of bulk erosion[17] . The molecular weight of all samples of microspheres decreased continuously after being exposed to PBS at 37°C. In all cases, the decrease in molecular weight almost follows a linear profile. Although a variety of definitions exists in the contemporary literature, it is well accepted that polymer degradation is defined as the process of polymer chain cleavage. Degradation is triggered by water which hydrolyzes the functional groups by which the monomers are usually connected. When the sample microspheres were exposed to PBS, water permeated into the microsphere matrix, resulting in random hydrolysis of ester bonds and decrease of the molecular weight[21].

Table 3: Molecular Weight For The Blanks Microspheres And For KSL Peptide Loaded Microspheres Before And After Incubation In Phosphate Buffer Saline pH 7.4 At 37°C.

Time (days)	M.wt. of RG503 microspheres	M.wt. of AcHES-RG503 microsphere
0 (Blank microspheres)	29427	27888
Loaded microspheres	27000	25555
	22517	22334
	19745	16667

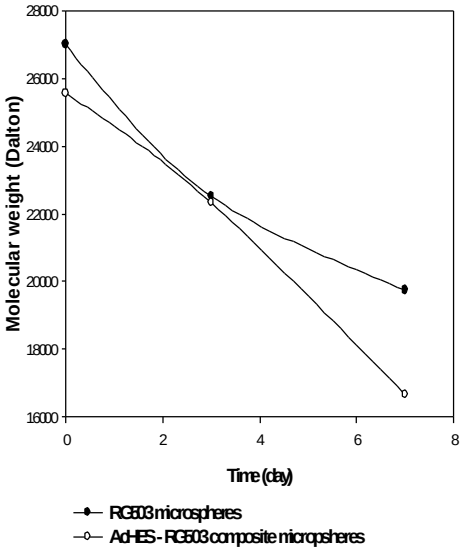

Figure 3:The molecular weight of 10%KSL peptide loaded microspheres before and after incubation in 0.1M phosphate buffer saline, pH 7.4 at 37°C.

Release of KSL Peptide Loaded PLGAs Microspheres:

A pathway for KSL peptide release was provided by microsphere degradation where water-soluble degradation products (i.e. monomers and oligomers) leave the microspheres matrix for the surrounding aqueous medium. Since oligomers are close to the surface they can leach out faster than that located deeper within the matrix, carboxylic acid oligomers trapped within the matrix autocatalyze further ester bond hydrolysis, resulting in the increasing rate of mass loss (22). Figure (4) shows the long-term in vitro release of the KSL peptide from KSL peptide loaded RG503, and KSL peptide loaded composite AcHES-RG503 microspheres. At 60 days, 69.12% of KSL peptide release were obtained from the microspheres and 82.32% from AcHES- RG503. Release kinetics (figure 4) of RG503 characterized by low burst release during 5 days period, followed by a continuous release for RG503 with no evidence of a lag phase. The release of KSL peptide from AcHES- RG503 composite microspheres in general was higher than that from RG503 microspheres. Dissolution of RG503 polymer in the composite microspheres could expose the entrapped KSL peptide-containing AcHES hydrogel particles to the release media, and the exposed hydrogel could release more KSL peptide molecules with little or no interaction with the PLGA polymer. A Similar behavior was observed by Capan et al; they reported that the composite microspheres showed more favorable in vitro release than conventional PLGA micropspheres for recombinant human growth hormone drug delivery (23). There have been attempt to improve protein stability and release kinetics of the PLGA system by changing the physicochemical properties of the polymer. For instance, both chemical derivation and physical bending of PLGA with hydrophilic polymers such as AcHES have been reported(24). AcHES hydrogel particles may have a protective function for enhancing the retention of KSL peptide stability and reducing KSL peptide adsorption to PLGA microspheres.

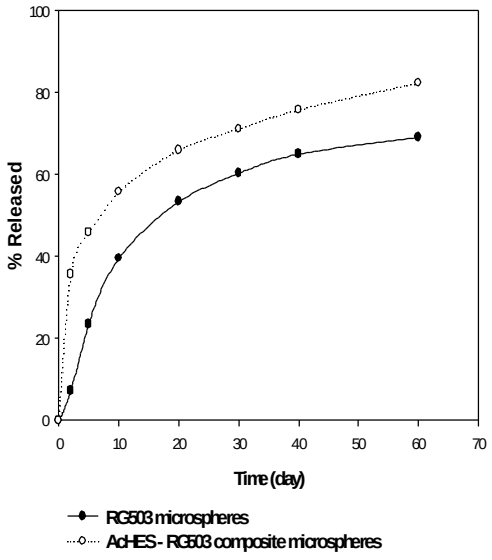

Figure 4: Release profile of 10% KSL peptide-loaded PLGAs microspheres in phosphate buffer, pH 7.4 at 37°C.

Conclusion :

The KSL peptide loaded PLGA microspheres and composite microspheres of a starch-based polymer and PLGA have been successfully formulated with spherical morphology, high capacity peptide incorporation efficiency, and good stability. The systems possesses sustained KSL peptide release for more than 60 days. The prolonged release of the KSL peptide may be potentially useful for long-term sustained release of the KSL peptide for the treatment of chronic bacterial and fungal infections

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