Preparation, Characterization and Prophylactic Study of New Microsphere Containing Doxycycline against Diseases of Shrimp

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Abstract

Therapeutically and prophylactically using Microspheres containing doxycycline isolated from shell of shrimp. Low molecule weight poly lactic acid was prepared. In this study, Poly lactic acid (PLA)/ poly vinyl alcohol (PVA)/poly ethyleneglycol(PEG) loading doxycycline blend solutions was prepared. Also Poly lactic acid (PLA)-Tannin blend via solvent evaporation method was prepared. Microspheres of chitosan/gelatin microsphere loading doxycycline was prepared by emulsion crosslinking technique. Both microsphere and blends were characterized by Fourier transform infrared (FTIR) spectrophotometer. The FTIR spectra were shown distinguish bands. The in *vitro* release of doxcycline from its matrix at pH 7 was studied. The prophylactic against white spot (Ich) disease of shrimp (*Macrobrachium nipponense*) was studied. The results were shown increase of percentage of survival of shrimp in both microsphere and blend compared with control. The highly percentage of survival was shown in the microsphere compare with blends.

Key words: Chitosan, Microsphere, Polymer blend, Shrimp, Prophylactic.

تحضير وتشخيص و دراسة وقائية للمايكروسفير المحمل بالدوكسيسايكلين لامراض الروبيان

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الخلاصـــة

يستخدم قشور الروبيان في تصنيع المايكروسفير المحمل بالدوكسيسايكلين للاغراض الوقائية والعلاجية , لذلك حضر بوزن جزيئي واطىء بولي حامض اللاكتيك. في هذه الدراسة أستخدم طريقة تبخير المذيب لتحضير مخاليط بوليمرية من بولي حامض اللاكتيك مع بولي فنيل الكحول مع بولي اثيلين كلايكول المحمل بالدوكسيسايكلين وكذلك حضر بولي حامض اللاكتيك مع المواد التانينية . وأستخدمت طريقة المستحلب المتشابك في تحضير المايكروسفير من الكيتوسان والجيلاتين والمحمل بالدوكسيسايكلين . شخصت المخاليط البوليمرية والمايكروسفير بواسطة طيف الاشعة تحت الحمراء ليعطي الحزم المميزة لهذه المركبات . درس تحلل الدواء من المنظومة البوليمرية في محلول منظم بدالة حامضية ٧ . وكذلك درس الخاصية الوقائية لمرض البقعة البيضاء المسببة لهلاكات الروبيان وقد اظهرت النتائج زيادة نسبة البقاء على قيد الحياة من الروبيان في كل من المايكروسفير والمخاليط البوليمرية مقارنة مع الكنترول وكانت نسبة البقاء الاعلى هي للروبيان المعامل بالمايكروسفير .

الكلمات المفتاحية: كيتوسان ،مايكروسفير ، مخلوط بوليمري ، روبيان ، وقائي .

Introduction

Microsphere plays an important role in the development of dosage forms for the health care industry, because the duration of drug release needs to be extended several days. The microsphere is incorporation of drugs into polymeric materials to control drug release and increase duration of action, the biodegradable polymers were used in drug delivery systems, control the release of therapeutic agents [(1)]. The polymer must biodegradable and does not require removal from the end of the treatment, because their degradation into physiological occurring compounds that can readily excreted. Polymer blends, that is, physical mixture of structurally different polymers which interact with secondary forces such as hydrogen bonding with no covalent bonding. Blending of three or more polymers has become an increasingly important technique for preparing materials with tailor made properties different from those of the constituent polymers. Blending of polymers may result in reducing their basic cost, improving their processing and maximizing their important properties. The increase inproperties of the blend depend on the degree of compatibility or miscibility of polymers at the molecular level [(2)].

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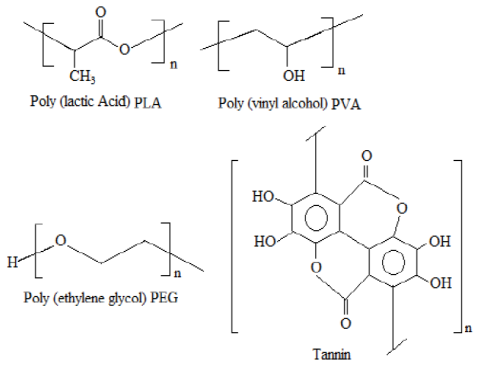
Received: 1/10/2014
Accepted: 5/4/2015

The biodegradation of polymer provides significant benefits such as reduction of stress resulting from animal handling and reduction of cost. The natural polymers are the most attractive and commonly used biodegradable polymers such as chitosan, poly (lactic acid) (PLA), polyvinyl alcohol (PVA),poly ethyleneglycol (PEG) and Tannin (scheme 1).



Scheme (1): Chemical structure for PLA, PVA, PEG and Tannin.

The natural polymers are commercially available in different compositions and molecular weights which effect in degradation of the polymer [3, 4].The degradation designates the process of polymer chain cleavage and loss of molecular weight. Degradation induces the fragments of the material which is defined as mass loss of material when polymer chain cleavage [5].

There are two different erosion mechanisms have been proposed for degradation; homogeneous or bulk erosion and heterogeneous or surface erosion [6].

The fish suffer from both diseases and parasites. It defenses against disease which include skin and scales, when the mucus layer secreted by the epidermis that traps microorganisms and inhibits their growth. The fish is developing inflammatory responses that increase the flow rate of blood to infected areas and deliver white blood cells that may destroy the pathogens.

The special responses to particular pathogens recognized by the fish's body, is adaptative immune responses [7]. The vaccines have become used in aquaculture and ornamental fish, such as vaccines for furunculosis in farmed salmon and koi, herpes virus,(Virus Herpes Salmon)VHS, Ich(white spot) and whirling disease[8,9]. All fish carry pathogens and parasites. Usually this is at some cost to the fish. If the cost of pathogens or parasites is high so its. Characterized as a disease. The disease in fish is not understood well relates to aquaria fish [10].

Disease is affecting in fish mortality, especially in the young fish. The natural droughts or pollution or predators, can precipitate outbreak of disease [11]. There are four general types of fish ailments, bacterial infections, fungal infections, parasitic or protozoan infections, and physical ailments and wounds [12].

The most antibiotics have been used against many intracellular and extracellular disease of fish is chloromycetin or tetracyclins [13-16].

The aim of this study, is synthesis of both microsphere and blends loading doxycycline as drug delivery system to enhance the percentage of survival of shrimp.

Materials and Method

Materials

Chitosan was isolated from shrimp shell , doxycycline was supplied from national center of drug evaluation and biological research. All materials were supplied from Aldrich company, glutaraldehyde, dichloromethane, n-hexan, poly(vinylalcohol), lactic acid, paraffin, gelatin, acetic acid ,hydrochloric acid, sodium hydroxide ,sodium bicarbonate and tween-80.

Methods

Extraction and deacetylation of chitin [17]

The resources used to extract chitin are shrimp shell. The shells were scraped free of loose tissue, washed, dried, and grounded.

Demineralization was carried out in dilute HCl solution (7% v/v) at room temperature with stirring and their durations (24-72 hours). The solid fraction was washed with distilled water until neutral pH. The demineralized samples were dried.

(10% w/v) NaOH at 60 °C for 24 hrs used for deproteinization of chitin. Then the solution was washed with water to neutrality, then with hot ethanol. The chitin was dried at 50 °C. 10 gm of chitin was put into (50% w/v) solution of NaOH at 60°C for 8 hours to prepare chitosan. The residue was washed with hot distilled water at 60°C for three times. The crude chitosan was drying in oven at 50°C overnight.

Preparation of poly lactic acid

Low molecular weight of poly lactic acid (PLA) was prepared [18]

Three necked flask equipped with mechanical stirrer, gas outlet tube, and nitrogen gas tube in bottom of flask, was charged with (0.13 mmole, 10 ml) of L-lactic acid , five drops of diluted 1% v/v HCl was add. The reaction mixture was stirred for 3 hours at 130°C and the nitrogen gas was passed through the mixture to get of the water. Then the reaction mixture was cooled and

neutralized by washing with 0.1 % w/v of $NaHCO_3$, then with water .The product was dried in vacuum-oven for 24 hours at 0.1 mm Hg and 40 °C to give a yellow oily product.

Preparation of chitosan/gelatin microsphere loading doxycycline **(19)**

Microspheres containing doxycycline were prepared by emulsion crosslinking technique. The chitosan, gelatin (1:1 by weight) and 0.5 gm ($1.133x10^{-3}$ mol) doxycycline were dissolved in 5% acetic acid. A certain amount of tween-80 and liquid paraffin at ratio 1:10 were added to the chitosan/gelatin mixture, under agitation was performed, using mechanical stirrer at 650 rev/min. at 30 °C.

After 15 minute, 4 ml of 25 % v/v glutaraldehyde solution was added drop by drop and stirring was continued for 3 hours. The microspheres so obtained were filtered and washed several times with n-hexane, then with distilled water to remove the adhering liquid paraffin and glutaraldehyde, respectively. The microspheres were dried in hot air oven at 50°C .

Preparation of microsphere by using Emulsification-solvent evaporation method **(20)**

1- Poly lactic acid (PLA) / polyvinylalcohol (PVA) /poly ethyleneglycol (PEG)-microsphere loading doxycycline

7.2 gm (0.1 mol) of (PLA) was dissolved in 20 ml dichloromethane. The solution was added drop-wise to a 40 ml aqueous phase solution containing 4.4 gm (0.1mol) (PVA), 4.4 gm (0.1 mol) (PEG) and 0.5 gm ($1.133x10^{-3}$ mol) of doxycycline was dissolved in polymer solution, while the mixture was stirred by an overhead stirrer to form a stable oil/water emulsion system at room temperature (25°C). Stirring was continued for overnight to allow the evaporation of both dichloromethane and water to form a solid blend. The blend was dried overnight in oven at 30 °C until no weight loss was observed. The melting point is 75°C.

2- Polylactic acid (PLA)-Tannin blend via solvent evaporation method

7.2 gm (0.1mol) of (PLA) was dissolved in 20 ml dichloromethane. The solution was then added drop-wise to 20 ml aqueous phase solution containing 30 gm (0.1mol) of tannin and 0.5 gm ($1.133x\ 10^{-3}$ mol) of doxycycline was dissolved in polymer solution. The mixture was stirred at room temperature (25°C), for two hours, to allow formation of solid blend. Then evaporation of dichloromethane and the blend was filtered , and dried overnight. The melting point is 210 °C (decomposition).

All microspheres and blend products were characterized by using FTIR spectro-photometer , (table -1).

Table (1): FT.IR spectral bands of products (cm^{-1}).

Products	**Streachin vibration υ CH_2**	**Streaching vibration υ O-H**	**νC=0 (Dox.)**	**νC=0**	**ν_{N-H} streaching vibration**	**νC-0**	**CH,CH_2 and CH_3 bending**
Chitosan/ gelatin microsphere.	as. 2923.88 s. 2858.31	3446.56	-------	1637	3250	1022.2	1458.08-1377.08
Chitosan/ gelatin- Dox. Microsphere	as. 2933.73 s. 2868.	3570.90	1620.04	1656.85 (acetylated chitosan)	3277	1118.71	1460.11-1348.24
PLA /Tannin-Dox. Blend.	as.2999.31, s. 2924 as.2954.95, s. 2852.72	3450	1618.28	1759.08 (tannin), 1720.5 poly(lactic acid)	3200 br	1188.15	1456.26-1386.82
PLA/PVA/PEG- Dox. Microsphere.	as 2949. 16 s. 2885.51 and as.2806.43 s. 2800	3616.53	1622	1755.51 (PLA)	3245	1114.86	1471.69-1361.74

Dox.=Doxycycline , as.= asymmetry , s. = symmetry. br. = broad

Release studies [21]

In vitro drug release from different formulations was invested in phosphate buffer solution (PBS) of pH 7.4. Microspheres (50 mg) were dispersed in 400 ml of PBS in a beaker and maintained at 37 ℃ under continuous stirring (60 rpm).At selected time intervals, 5 ml samples were withdrawn and replaced with the same volume of prewarmed fresh buffer solution to maintain a constant volume of the receptor compartment. The samples were analyzed spectrometrically at 347 *nm* (dilution factor 10). The released drug content was computed from the calibration curve of doxycycline as shown in figure 6.

Application of both microspheres as prophylactic agents

Collect the shrimp sample (*Macrobrachium nipponense*) from Shat Al-arab , Qarmat Ali in 7/4/2014, the small size shrimp was chosen with average weight 0.5±0.1 gm and average height 26± 2 mm. In this experiment we used four plastic aquariums with capacity of 15 liters for each one, in first aquarium treated with chitosan/gelatin microsphere containing doxycycline, and in the second aquarium treated with PLA/PVA/PEG –Doxycycline microsphere, while the third one was treated with PLA/Tannin –Doxycycline blend. In the last one, left without antibiotic. The addition of microsphere is 0.25 gm every 48 hours and the water replacement occur in partial manner, during study, the environmental data are recorded through four weeks and the result as follow temperature, in 23-25°C, salt is 1.2-1.5 gm/L, dissolved O25.5-6.4 mg/L .

Results and Discussion

Microsphere is drug delivery offers an intelligent approach in the medical field. It is encapsulating a drug into a carrier particle [22]. Biodegradable microsphere can be prepared from certain synthetic, as well as natural polymers. Natural polymers remain attractive primarily because they are natural products of living organisms, readily available, relatively inexpensive and non-toxic. Therefore, using natural polymers such as gelatin and chitosan figure (1), have many benefits for developing successful drug delivery systems. The microspher provides sustained release, targeting and stabilization of drug [23-25].

From much research doxycycline application is limited by its relatively low stability in aqueous solution. Therefore, to solve this problem by using microsphere to form inclusion compounds within molecules, show great advantages in enhancing the stability of guest molecules [26].

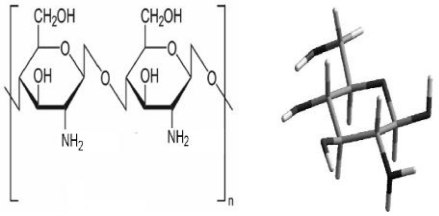


Figure (1): Chemical and 3D-structure of N-D-glucosamine(chitosan).

The incorporation between drug and polymer was studied by FTIR spectroscopy. Spectral-data were recorded for drug-loaded microspheres and blend types. Table1, illustrate the main characteristic bands in the products.

The comparative FTIR spectra of doxycycline -loading microspheres and blend types are shown in Figures (2-4). Peaks are observed around 2900-3000 cm^{-1} corresponding to aliphatic -CH and -CH_3 of the polymers, chitosan, gelatin, PLA and PVA. The result indicates the stability of the drug during the microencapsulation process, also showed small deviations of some polymer bands like from 1637 (without drug) to 1656.85 cm^{-1}, probably due to the presence of doxycycline

Figure(2): FT.IR Spectrum of Chitosan/ Gelatin –Doxycycline microsphere

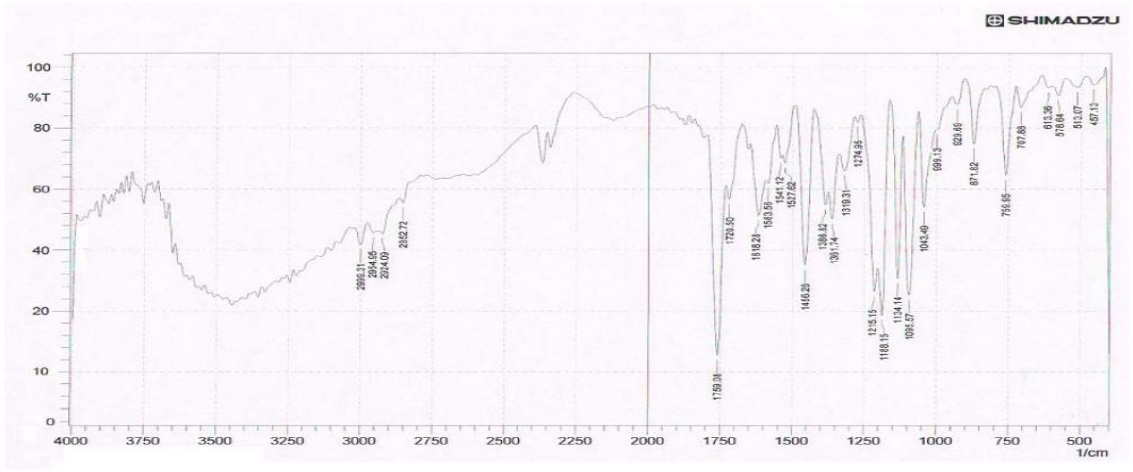
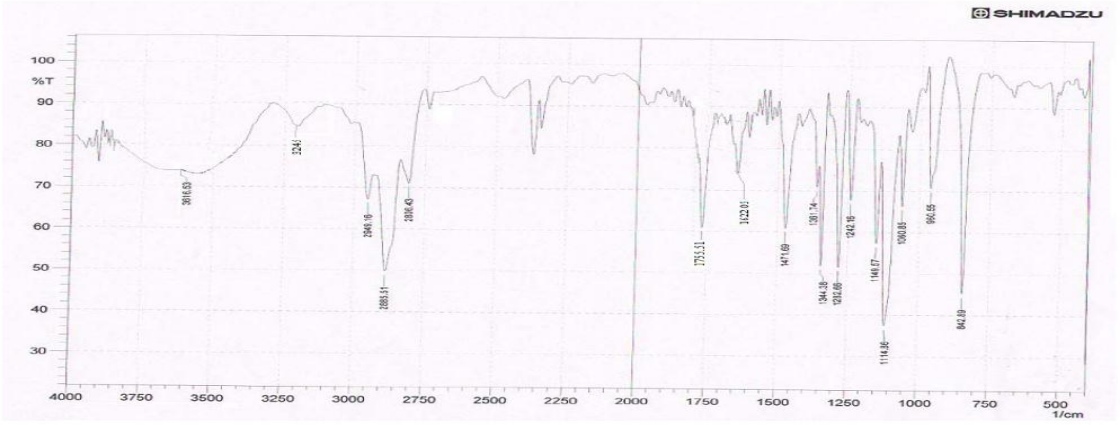


Figure (3): FT.IR Spectrum of Tannin /PLA-Doxycycline blend.



Figure(4) : FT.IR Spectrum of PLA/PVA/PEG-Doxycline microsphere.

Extensive research has been carried out to exploit the use of chitosan as a drug or vaccine carrier [27-29]. The safety of chitosan has been extensively studied, and it was found that it is a biologically compatible polymer with a minimal toxicity [30, 31] .

The *in vitro* release profiles of doxycycline formulations as microspheres, and blend, are shown in Figure 5. The results illustrated, that the drug release is fast and requires peroid of time to go completion. This can be explained by the high affinity of the drug towards water, used as a release medium. The release rate is also higher for formulations having PLA / Tannin -doxycycline blend and PLA /PVA /PEG -Doxycyline, than chitosan/gelatin-Doxycycline microsphere.The formation of particular aggregates, which extends into the polymer matrix, needs more time to dissolve. The initial release observed for PLA/Tannin-Doxycycline can be explained by the presence of surface associated drug. The aggregates due to the small quantity of drug, leads to fast dissolution into the release medium during the first minutes, especially in the case of hydrophilic drugs[32].

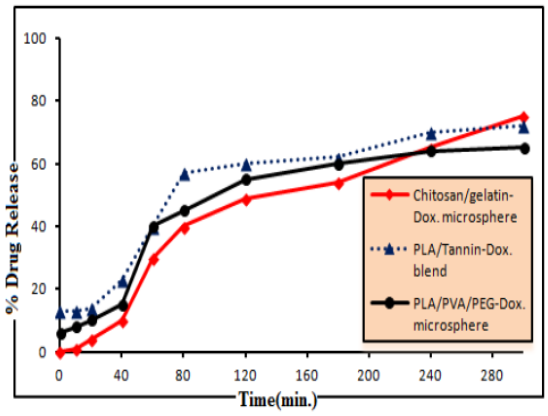


Figure (5): Release of doxycycline from products.

Bacterial diseases are usually characterized by red streaks or spots and/or swelling of the abdomen or eye. These are treated by antibiotics such as doxycycline, which has been successfully encapsulated through the microspheres. Then investigation both microsphere types, are more effective than blend type, from percentage of survival of shrimp. Table 2 shows the high percentage of

survival to the shrimp sample of the treated groups, compared with the control.

Table (2) Percentage of survival to the shrimp sample

Treatment	% of survival
Chitosan/gelatin – Doxycline Microsphere	97
PLA/PEG/PVA-Doxycycline Microsphere	90
PLA/Tannin-Doxycycline blend	89
Control	78

Conclusion

Doxycycline has been successfully encapsulated in microspheres and applied as prophylactic in shrimp culture to control release of doxycycline. The results showed that drug release is fast and only few hours to go to completion were required. This can be explained by the high affinity of the drug towards water used as a release medium. Then, the release rate is higher for formulations having the low amount of drug .The conclusion that, at more drug loading, the high chance of particles to interact with each other. This leads to, the formation of particular aggregates, which extends into the polymer matrix, and needs more time to dissolve.

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