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양식장에 적합한 키토산 부유 미립구의 제조

朝鮮大學校 大學院

藥 學 科

金 鎭 宇

양식장에 적합한 키토산 부유 미립구의 제조

Preparation of floating microsphere of chitosan for fish farming

2006年 2月 日

朝鮮大學校 大學院

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양식장에 적합한 키토산 부유 미립구의 제조

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At the first step of my academic itinerary, this thesis for Masters degree will be recorded as a maiden work.

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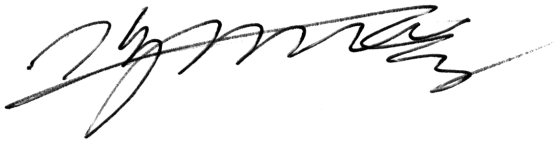
Stay Hungry! Stay Foolish!

So help me God!

Dec. 3, 2005



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양식장에 적합한 키토산 부유 미립구의 제조

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물고기 양식업은 많은 양의 물고기를 생산함으로써 영양 식품에 대한 수요를 보다 저렴한 가격으로 충족시켜 주기 때문에 그 추세가 계속 증가해 왔다. 그러나, 박테리아나 곰팡이에 의해 발생하는 진균증과 같은 다양한 감염성 질환은 양식장에서 키우는 물고기의 공급량을 감소시키는 심각한 문제로 대두되어 왔다. 이러한 문제점을 극복하기 위해 flumequine이나 oxytetracycline과 같은 많은 항생제들을 사용하여 양식장에 있는 박테리아 병원균들을 억제하거나 예방하려는 시도가 행해져 왔다. 현재 양식장에서 사용되고 있는 몇 가지 항생제 투여 방식이 있는데, 먼저 항생제를 음식과 함께 투여하는 방법이 있다. 또한, 약물을 섞은 양식장의 물에 물고기를 수욕 시키거나 약물 자체를 물고기가 살고 있는 물에 녹이기도 한다. 그러나, 이러한 투여 방식들이 반드시 바람직하거나 효과적이지는 않다. 약물을 음식과 같이 혼합하여 물고기에게 먹일 때 항생제가 물고기에게 투여될 확률의 편차가 물고기가 섭취하는 음식물의 양에 따라 심할 뿐 아니라 항생제 중에서 위에서 언급한 macrolide 계열의 항생제들은 친수성이 작기 때문에 물 아래로 침전되기 쉽다. 이처럼, 투여된 약물의 침전되는 것과 같이 물고기가 섭취하지 못해 발생하는 손실을 감안하여 약물을

과량 사용할 수밖에 없다. 그러나, 대부분의 항생제가 물고기의 체내에서 축적되지 않고 대사 및 배설이 된다 하더라도, 그것을 과량 사용하게 될 때 일부 약물이 체내에 잔류할 수 있고 이는 물고기에게 부작용을 초래할 수 있다. 예를 들어, oxytetracycline은 무지개 송어의 체내에 잔류할 경우 항체 반응을 억제한다고 알려져 있다. 이러한 잔류 기간을 고려하여 대부분의 양식장에서는 항생제를 투약한 후 휴약 기간을 둔다. 또한, 약물로 인하여 변형된 물고기는 그것을 섭취한 사람들에게도 직접적으로 치명적인 영향을 끼칠 수 있다. 양식업에서 가장 많이 사용되는 tetracycline 계열의 약물은 위장관과 간, 신장 그리고 치아에 독성을 일으킬 수 있다. 더욱이, 항생제의 남용은 최소 억제 농도와 최소 살균 농도를 증가시킴으로써 박테리아의 약물에 대한 내성을 강하게 하고 이것은 항생제의 효능을 저하시킨다. 그러므로, 적은 양의 항생제를 가지고 물고기의 감염증을 예방, 치료하는 것은 위에서 기술한 관점에서 볼 때 바람직할 것이다. 왜냐하면, 이러한 투여 방식은 약물의 손실이나 낭비를 최소화시키면서 물고기의 약물 흡수를 최대화시키는 것이기 때문이다. 따라서, 특별한 운반체를 사용하여 항생제의 제어 방출 제형을 개발하는 것은 의미 있는 분야이자 시도라고 할 수 있다. 제어 방출과 같은 약물 송달 시스템을 통하여 운반체에 적절한 양의 항생제를 적재시키고 특정 부위에서 적정 속도로 그것을 방출시킬 수 있다.

본 연구에서는 물고기 양식업에 적합한 약물 송달 운반체로서 키토산 부유 미립구를 선택했다. 아세틸 글루코사민과 글루코사민이 β(1→4) 결합을 하고 있는 천연 중합체의 하나인 키토산은 생체 적합성과 생체 분해성과 같은 여러 장점을 가지고 있기 때문에 키토산을 이용한 particulate system은 약제학과 의공학 분야에서 많이 연구되었다. 특히, 키토산은 산성 조건에서 용해도가 높고 팽창이 잘되는 특이한 성질을 갖고 있기 때문에 양식장에서 이용할 수 있는 항생제 제형을 만들어서 경구용 약물 송달 시스템을 개발하고자 하는 본 연구의 주제에 적합하다. 키토산 미립구는 다공성 표면 구조를 갖고 있어서 물 위에서 부유할 수 있기 때문에 미립구에 적재된 약물이 침전되지 못하게 할 수 있다. 반면, 물고기의 위와 같은 산성 조건에서는 미립구가 팽창된 후 용해되어 약물을 방출시킬 수 있다. 이러한 목표를 얻고자 우리는 다공성 구조를 갖는 키토산 부유 미립구를 에멀젼화-용매 증발법으로 제조했다. 또한, 미립구 제조의 최적화 조건을 찾는 실험을 수반했다. 물고기의 항생

제로써 Oxytetracycline · HCl을 제조된 키토산 미립구에 적재했는데, 이 약물은 특히 무지개 송어에 많이 사용되며 낮은 산성 조건에서도 안정성과 물리화학적 성질을 유지한다고 알려져 있다. 미립구의 부유 실험과 약물 방출 실험을 통하여 키토산 미립구가 양식장에 적합한 지 알아 보았다. 또한, 무지개 송어의 양식업이 fish farming market에서 상당히 큰 비중을 차지하고 있다는 관점에서 이 실험의 의의를 찾을 수 있겠다.

1. Introduction

Recently, artificial fish farming has greatly increased because it can supply human with enough fishes to satisfy demands on cheaper nutrients. However, various infective diseases of fishes such as mycobacteriosis caused by bacteria or fungi have been one of the most serious problems in decreasing the supplying amounts of fishes which have been culturally raised (12, 17). To recover this problem, many antibiotics such as flumequine, oxytetracycline have been widely used for fish bacterial diseases in preventing or controlling bacterial pathogens in fish farming (1, 2).

There are some types of antibiotic administrations used presently. One is the oral administration of drugs as food additives (17, 21). Another is to bathe fish inside aquaculture medium containing drugs. The third is the spraying method by which drugs are dissolved in medium. However, these kinds of administrations do not always bring desirable and profitable results. When drugs are medicated with food, the exposure dose of antibiotics can be varied depending on the different amounts of fish diet which are ingested. Also, antibiotics, especially macrolide class of antibiotics mentioned above, can be precipitated (21) because of their low hydrophilicity (11). These issues are related to the overuse of those drugs for the purpose of supplementing some loss of administered amounts. As well, this plethora medication can give fish toxic effects, even though most antibiotics are metabolized and excreted without accumulation in fish. For instance, oxytetracycline is known to suppress the antibody response on rainbow trout if remained inside the fish (17). Considering this remaining duration, the medication of antibiotics in most fish farming places needs the withdrawal periods of drugs (17). Also, this kind

of altered fish can directly have deleterious effects on human if ingested. Tetracycline, one of the most widely used classes of antibiotics in fish farming, can give toxic effects to gastrointestinal tract, liver, renal organs and teeth (13). Moreover, bacteria resistance to antibiotics which must have been formed by increasing MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) can incapacitate the efficacy of most antibiotics against bacterial pathogens (11, 14). Undoubtedly, it is the most desirable for antibiotics with small dose to prevent or treat fish infection in respect of some reasons above, minimizing the loss or extravagance of drugs disseminated on freshwater and maximizing the absorption of drugs into fish. In this meaning, the development of controlled release dosage form using specific carrier can be issued interestingly because that kind of drug delivery system enables antibiotics to be loaded inside the carrier with moderate amount and to be released with intentional rate at only specific targeted sites (15, 16).

In this study, floating microsphere of chitosan was selected as controlled drug delivery vehicle for fish farming. Because chitosan, a kind of natural linear copolymers of β-(1,4)-linked N-acetylglucosamine and glucosamine, has several advantages such as biodegradable and biocompatible properties, chitosan-based particulate systems have already attracted pharmaceutical and biomedical applications as potential drug delivery devices (10).

Especially, singular characteristics of high solubility and swelling properties of chitosan by protonation and hydration of its amino groups in acidic condition encouraged us to study appropriate oral drug delivery system by formulating antibiotics utilized in fish farming (4, 7, 8). Chitosan microsphere is expected to prevent loaded drugs precipitating down freshwater because it is expected

that the low density caused from the porous structures of prepared microspheres (8) can contribute to their floatability. Whereas, it swells and dissolves in acidic condition such as the interior of fish stomach resulting in the releases of drugs.

For the sake of this object, we conducted this study to develop chitosan microspheres with pores using emulsification-solvent evaporation method. Also, the preparation conditions were optimized. Oxytetracycline·HCl as a model antibiotic for fish, especially rainbow trout (20, 21), was loaded into chitosan microsphere. Oxytetracycline·HCl is known to keep its stability and physicochemical properties in low pH condition (16). Also, a few studies including floating and drug release tests necessary to the development of drug delivery suitable for aquaculture were conducted. Also, because the aquaculture of rainbow trout has occupied considerably the world market of aquaculture industry (17), this study is very meaningful in that the creation of sufficient profits and efficiency in the field of fish farming can be expected.

2. Materials and methods

2-1. Materials

Chitosan (low molecular weight, Brookfield viscosity : 20.000 cps) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Sorbitan monooleate (Span®80) was obtained from Junsei Chemical Co. (Tokyo, Japan). Acetic acid and ethanol were purchased from Merck Chemical Co. (Darmstadt, Germany) and oxytetracycline·HCl was bought from Sigma Chemical Co. (St. Louis, MO). Corn oil was purchased from CJ Corporation (Seoul, Korea). All other chemicals were grade of reagent available commercially.

2-2. Methods

2-2-1. Preparation of Chitosan Microspheres

The chitosan microspheres were prepared by emulsification-solvent evaporation method. Briefly, chitosan was dissolved in 2 % acetic acid solution. After the complete dissolution of chitosan, ethanol was added. To prepare w/o emulsion, Span®80 as a w/o emulsifier was added to corn oil as an external phase and stirred for 10 min. Chitosan solution fabricated above was then added dropwise but simultaneously with syringe needle to the external phase containing Span®80. This final solution was kept on stirring for 2 days until solvents in water phase completely partitioned into the oil phase and were removed by evaporation. Rinsing this solution with excess hexane and filtering it with reduced pressure were repeated two times. These ultimately formed microspheres were dried at 60℃ in a vacuum dryer for 12 h. On the purpose of finding optimal conditions in preparing microspheres, several

factors such as internal phase volume fraction, solvent ratio of internal phase, surfactant concentration, stirring speed and preparation temperature were considered. In the study of internal phase volume fraction, other parameters included 1 % polymer concentration, room temperature, 500 rpm stirring speed, 7:3 (ethanol : water) of solvent ratio in the internal phase and 0.4% surfactant concentration. As the experiment for optimization proceeded and each parameter was determined, subsequent experiment carried the newly determined parameter.

2-2-2. Preparation of Oxytetracycline·HCl (OTC) loaded Chitosan Microspheres

All procedures were the same as those of chitosan microsphere except adding the drug (5 % of the total amount of chitosan).

2-2-3. Yield of formed Microspheres

The yield was calculated by dividing the obtained amount of microspheres by the total amount of all the non-volatile components used for preparing microspheres. It was supposed that other components were all evaporated except chitosan and acetic acid. Because it is known that one unit of chitosan and acetic acid can form complex with 1:2 molar ratio (5), the total amounts of non-volatile components was obtained by adding the amount of chitosan needed to form such complex to that of chitosan used for preparing microspheres.

2-2-4. Particle Size Analysis

The particle size of the microspheres was determined by sieving analysis using standard sieves (Chung-gye Industrial MFG., Korea).

2-2-5. Scanning Electron Microscopy (SEM)

The morphology of the microspheres was examined by field emission scanning electron microscopy (S-4700, Hitachi, Japan). The samples were mounted onto an aluminum stub and sputter-coated for 120 s with platinum particles in an argon atmosphere.

2-2-6. Release study of OTC from the Microspheres

This study was carried out at simulated fish gastric solution of pH 2.7 (rainbow trout) at room temperature. After 60 mg of OTC-loaded chitosan microspheres was placed in tea-bag which was then attached on the side wall of beaker, 200 ml of pH 2.7 solution as release medium was poured and stirred at 100 rpm. An aliquot (1 ml) of the release medium was withdrawn at predetermined time intervals and an equivalent amount of fresh medium was added to the release medium to maintain sink condition. The collected samples were filtered through a 0.45 μm-syringe filter and analyzed using HPLC at 353 nm and the quantity of OTC released from the microspheres determined.

2-2-7. Floating test

The purpose of preparing floating chitosan microspheres is to prevent them from settling down at freshwater and to keep their buoyancy until absorbed in fish. The floating test was carried out to investigate the floatability of the prepared microspheres. Chitosan microspheres (50 mg) were spread over the

surface of the dispersing medium (900 ml of freshwater) at 100 rpm at room temperature for 12 hr. After removing them floating on the surface, the precipitated microspheres were collected at predetermined time points. The collected samples were weighed after drying.

2-2-8. Loading efficiency

After 20 mg of OTC-loaded microspheres were completely dissolved with 10 ml of 5 % acetic acid for 20 hr, the amount of loaded drug was analyzed by HPLC. Loading efficiency was calculated by comparing the amount of the drug used to prepared microspheres with that of the drug loaded.

2-2-9. HPLC analysis

Analysis condition was determined according to *P. Chetoni et al.* with a little modification (22). The apparatus (Shimadzu Scientific Instruments, MD, USA) consisted of a UV detector (SPD-10A), a pump (LC-10AD), an automatic injector (SIL-10A) and a column (Waters, Symmetry®C8 5μm, 4.6×150mm) to determine the amount of OTC released from the microspheres. The wavelength of the UV detector was 353 nm. The column temperature was maintained at 37°C. The flow rate was 1 ml/min and the mobile phase was composed of methanol/water (40/60), triethylamine (1 %) and phosphoric acid (0.8 %).

3. Results and discussion

3-1. Preparation of Chitosan Microspheres

Chitosan dissolves well in acetic acid because amino groups of chitosan can be ionized positively by hydrogen ion of acetic acid (2, 3). To this chitosan solution, specific amount of ethanol was mixed to shorten the preparation time. The worry about toxic effects of residual solvents in microspheres can be clearly relieved by the use of ethanol as a co-solvent because ethanol itself is not a seriously toxic solvent and it can be nearly evaporated at room temperature (24). After the internal phase was dispersed in corn oil, the droplets were gradually solidified and hardened as the ethanol and water were diffused out of the internal phase and evaporated (24, 25).

In order to identify the optimal preparation conditions, the effects of various experimental parameters including internal phase volume fraction, preparation temperature, solvent ratio of the internal phase, stirring speed and surfactant concentration on the formation of microspheres were investigated.

3-1-1. Effect of Internal Phase Volume

The volume fractions of the internal phase tested were 5, 7, 9, 11 %. The yield of the chitosan microspheres formation is shown in Table 1. The yield generally increased as the internal phase volume incremented. Because it is difficult to separate aggregated particles from microspheres, the microparticles are included in the yield measurement. Therefore, the actual yield of the microspheres would be much lower than the measured value. At higher internal phase volume, the degree of aggregation tends to strengthen. As the volume fraction of the internal phase increased, the medium became

densely populated with internal droplets. Until ethanol and water inside the droplets were completely diffused out and evaporated, the formed microspheres would be sticky. Due to the dense population and sticky property of the droplets, they have a greater chance of coming in contact with each other and aggregating. Therefore, as the volume fraction of the internal phase exceeds the optimal range, larger droplets or aggregates tend to form.

3-1-2. Effect of Preparation Temperature

The temperature of the dispersing medium is an important factor in the formation of microspheres because it controls the evaporation rate of the solvents. The investigation on the effect of temperature was carried out at room temperature, 40 and 60°C. Microspheres at 40 and 60°C had irregular shapes. Especially, microspheres at 60°C formed a large aggregate on the upper center of external phase during preparation and finally appeared to be streamline. It might be caused by faster diffusion of solvents in the droplet into oil phase and evaporation immediately after introducing it into the medium (3). Over all, the yield decreased with the increase in preparation temperature. The optimal temperature for good microspheres was evidenced as room temperature with the highest yield.

3-1-3. Effect of Stirring Speed

Table 1 shows the effect of the stirring speed on the formation of microspheres and particle size. As the stirring speed increased, the mean particle size decreased. At 300 rpm, the droplets could not disperse in the corn oil actively and were formed as films covered on the oil phase for long time, which elongated the hardening time because the films inhibited the

evaporation of solvents. Accordingly, the remaining solvents by such inhibition caused considerable amounts of microspheres to be adhered to the walls and bottoms of beakers. At 400 rpm, after chitosan solution which was dropped into continuous phase was initially formed as large emulsions, such large droplets were finally taken into smaller broken microparticles. In addition, less than 500 rpm could not keep the morphology of formed microspheres as regularly and spherically as 500 rpm. Stirring provides the energy to break up the emulsion droplets and it is obvious that smaller droplets will be formed as the stirring speed increases as a result of the high shear induced by high stirring speed (24). Actually, the particle size was significantly reduced as the stirring speed increased from 300 to 500 rpm.

3-1-4. Effect of Solvent Ratios of Internal Phase

The effects of the various solvent ratios of the internal phase (ethanol/water) on the formation and the particle size of microspheres were investigated (Table 1). Over an ethanol/water ratio of 7/3, chitosan hardly dissolved in the mixed solvent. The content of water in the internal phase appears to play a key role in the formation of microspheres. After ethanol is preferentially diffused out into the corn oil phase, water mainly constitutes the core of the emulsion droplet (25). Actually, the mean particle size increased as the solvent ratio got lower. However, at the ratios of 5/5 and 4/6, the microspheres showed irregular shapes and some of them were aggregated. The water content directly affects the solidification time of the microspheres. At 5/5 and 4/6, the solidification time of the microspheres increased due to the relatively large amount of water, which increased the collision frequency between the incompletely solidified microspheres with adhesive properties in

the wet state. Accordingly, some were aggregated or irregular because of frequent collision. Because larger droplets tend to aggregate and smaller droplets form microspheres, the size of microspheres at 5/5 and 4/6 was rather decreased even if the portion of water increased.

3-1-5. Effect of Surfactant Concentration

The effects of the surfactant (Span®80) concentration in the corn oil on the formation of the microspheres and the particle size are shown in Table 1. The yield generally increased with the surfactant concentration except 1.6 %. Also, the microspheres at 1.6 % showed some irregular forms. When 0.2 % of Span®80 was used, considerable microspheres were broken or aggregated with needle-shaped particles. This is why the droplets without or with a little surfactant tend to be collapsed or cohered after colliding between themselves. When 1.2 % of Span®80 was used, the microspheres was the most spherical with good yield.

3-2. Morphology

The morphology of the finally optimized microspheres was examined by SEM. Their views showed spherical shapes with smooth surfaces (Fig. 1-a, b). Also, the shells of the microspheres showed some porous structures. It may be caused by the evaporation of solvents entrapped within the shells of microspheres after forming smooth and dense skin layers (3, 24).

3-3. Release study of OTC from the Microspheres

Fig. 2 shows the in vitro release profiles of OTC from the microspheres at pH 2.7 solution at room temperature for 18 h. As seen, significant amount of

drug was abruptly released at pH 2.7 solution within 30 min. This result of release study convinced us that chitosan microspheres can deliver OTC into rainbow stomach and release it there.

3-4. Floating test

As shown in Fig. 3, the fractions of microspheres floating on the medium showed almost horizontal line up to 12 hr, keeping the floatability from 60 to 70 % except the initial sudden precipitation. Hence, it can be suggested that these chitosan microspheres can resist on precipitating until fish can ingest them. Also, it is not needed to fear that significant amounts of loaded drugs might diffuse out from that carrier because this schemed formulation has very small contact surface with the medium when floating (8). In other words, it seldom gets wet or hydrated by the medium (8). Also, those porous spaces of chitosan microspheres are too small for medium to invade would be instead filled with air. Above all, this can ensure high possibility that fish is capable of biting and ingesting drugs.

3-5. Loading efficiency

It is clear that the solubility of the drug will determine the preferential location of the drug among the solvents used (3, 24). When the drug is more soluble in ethanol than in water, the drug may be diffused out from an emulsion droplet with ethanol before the droplet solidification, leading to low loading efficiency. It is well known that OTC has much higher solubility in water (1 g/ml) than in ethanol (12 mg/ml) (16). Final loading efficiency was estimated as 80.3±5.7 % through HPLC analysis. Thus, the high loading efficiency of OTC can be attributable to its relatively low solubility in ethanol.

4. Conclusions

It is evident that chitosan microsphere is eligible for fish farming as oral delivery of OTC through this study. Also, this delivery system was proven to be enough desirable to solve some problems steadily issued in current methods of antibiotics administration to fish even though some studies such as release study on freshwater are further needed. Especially, because some environmental parameters such as water source, water temperature and pH are various depending on the kinds of farmed fishes, it is expected that the development of microsphere formulation can be applied to other fishes

References

1. G.M. Lalumera, D. Calamari, P. Galli, S. Castiglioni, G. Crosa and R. Fanelli, 2004, Preliminary investigation on the environmental occurrence and effects of antibiotics used in aquaculture in Italy, *Chemosphere* 54(5), 661-668.

2. K.L. Shantha, D.R.K. Harding, 2002, Synthesis and characterization of chemically modified chitosan microspheres, *Carbohydrate Polymers* 48, 247-253.

3. J.H. Lee, T.G. Park and H.K. Choi, 1999, Development of oral drug delivery system using floating microspheres, *J. Microencapsul.* 16, 715-729.

4. V.R. Sinha, A.K. Sigla, S. Wadhawan, R. Kaushik, R. Kumria and K. Bansal et al., 2004, Chitosan microspheres as a potential carrier for drugs, *Int. J. Pharm.* 274, 1-33.

5. J. Nunthanid, M. Laungtana-anan, P. Sriamornsak, S. Limmatvapirat, S. Puttipipatkhachorn, L.Y. Lim, E. Khor, 2004, Characterization of chitosan acetate as a binder for sustained release tablets, *J. Control Rel.* 9, 15-26.

6. R.K., Huang, M.Y. and Jiang, Y.Y., 1988, Selective permeabilities of chitosanacetic acid complex membrane and chitosanpolymer complex membrane for oxygen and carbon dioxide, *Polym. Bull.* 20, 83-88.

7. S.-M. Cho, H.-K. Choi, 2005, Preparation of mucoadhesive

chitosan-poly(acrylic acid) microspheres by interpolymer complexation and solvent evaporation method, *Arch Pharm Res* 28(5), 612-618.

8. El-Gibaly. I., 2002, Development and in vitro evaluation of novel floating chitosan microcapsules for oral use: comparison with non-floating chitosan microspheres, *Int. J. Pharm.* 249, 7-21.

9. B. Carreno-Gomez and R. Duncan, 1997, Evaluation of the biological properties of soluble chitosan and chitosan microspheres, *Int. J. Pharm.* 148, 231-240.

10. S.A. Agnihotri, N.N. Mallikarjuna and T.M. Aminabhavi, 2004, Recent advances on chitosan-based micro- and nanoparticles in drug delivery, *J. Control Rel.* 100(1), 5-28.

11. S.D. Costanzo, J. Murby and J. Bates, 2005, Ecosystem response to antibiotics entering the aquatic environment, *Mar. Pollut. Bull.* 51(1-4), 218-223.

12. R. Belas, P. Faloon and A. Hannaford, 1995, Potential applications of molecular biology to the study of fish mycobacteriosis, *Annu. Rev. Fish Dis.* 5, 133-173.

13. Joel G. Hardman, Lee E. Limbird, Goodman&Gilman's the pharmacological basis of therapeutics (10th edition).

14. P.R. Smith, M.P. Hiney and O.B. Samuelsen, 1994, Bacterial resistance to

antimicrobial agents used in fish farming : a critical evaluation of methods and meaning, *Annu. Rev. Fish Dis.* 4, 273-313.

15. Y. Sato, Y. Kawashima, H. Takeuchi and H. Yamamoto, 2004, In vitro evaluation of floating and drug releasing behaviors of hollow microspheres (microballoons) prepared by the emulsion solvent diffusion method, *Eur. J. Pharm.Biopharm.* 57(2), 235-243.

16. The Merck Index (Twelfth edition).

17. Robert. R. Stickney(editor), Encyclopedia of aquaculture, Wiley-interscience, NY.

18. A. Lindberg and G. Olivecrona, 2002, Lipoprotein lipase from rainbow trout differs in several respects from the enzyme in mammals, *Gene*, 292(1-2), 213-223.

19. Dauble, D.D. and Curtis, L.R., 1990, Influence of digestive processes on the absorption and fate of quinoline ingested by rainbow trout(oncorhynchus mykiss), *Environ. Toxicol. Chem.* 9, 505-512.

20. H. Bjorklund, J. Bondestam and G. Bylund, 1990, Residues of oxytetracycline in wild fish and sediments from fish farms, *Aquaculture* 86, 359-367.

21. A. Rogstad, V. Hormazabal, O.F. Ellingsen and K.E. Rasmussen, 1991,

Pharmacokinetic study of oxytetracycline in fish : 1. Absorption, distribution, and accumulation in rainbow trout in fresh water, *Aquaculture*, 96(34), 219-226.

22. P. Chetoni, G. Di Colo, M. Grandi, M. Morelli, M.F. Saettone and S. Darougar, 1998, Silicone rubber/hydrogel composite ophthalmic inserts : preparation and preliminary in vitro/in vivo evaluation, *Eur. J. Pharm. Biopharm.* 46, 125-132.

23. G. Crotts and T.G. Park, 1995, Preparation of porous and nonporous biodegradable polymeric hollow microspheres, *J. Control Rel.* 35, 91-105.

24. P.B. O'Donnell and J.W. McGinity, 1997, Preparation of microspheres by the solvent evaporation technique, *Advanced Drug Delivery Reviews* 28, 25-42.

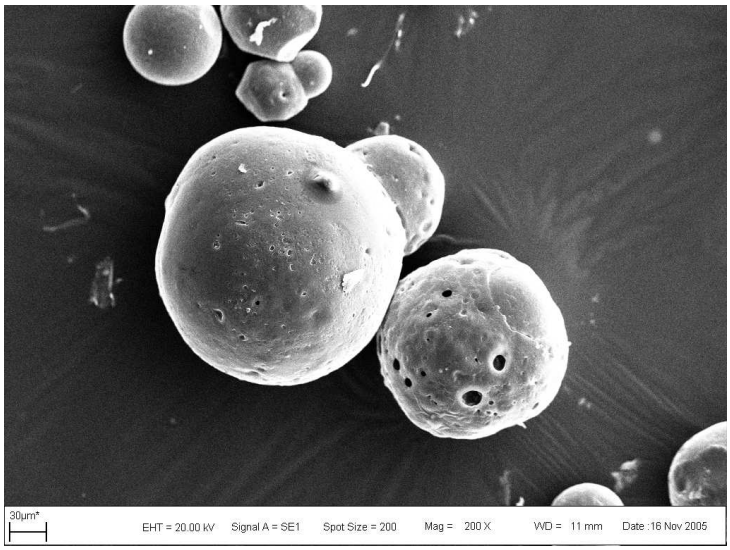
25. M.-K. Chun, C.-S. Cho and H.-K. Choi, 2005, Mucoadhesive microspheres prepared by interpolymer complexation and solvent diffusion method, *Int. J. Pharm.* 288, 295-303.

Table 1

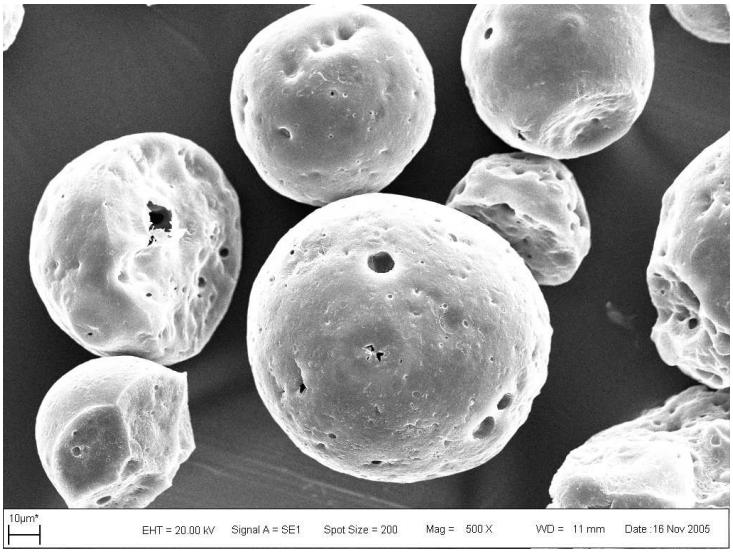
Effect of various parameters on the formation and mean particle size of the microspheres (n=3)

Processing parameter	Mean particle size (㎛) (mean±S.D.)[a]	Yield (%) (mean±S.D.)[a]
Internal phase volume fraction		
11 %	278.4±54.8	72.3±8.8
9 %	134.1±32.1	65.5±0.8
7 %	152.2±31.6	51.8±2.6
5 %	95.9±23.1	37.0±0.6
Preparation temperature		
60℃	154.1±28.9	29.6±0.8
40℃	249.9±48.2	53.6±3.8
Room temp.	134.1±32.1	65.5±0.8
Stirring speed		
500 rpm	134.1±32.1	65.5±0.8
400 rpm	335.4±12.2	61.8±2.9
300 rpm	-	-
Solvent ratio of internal phase (ethanol/water)		
7/3	134.1±32.1	65.5±0.8
6/4	269.7±38.6	61.9±3.8
5/5	232.2±20.9	69.2±4.2
4/6	153.2±36.8	77.9±5.1
Surfactant (Span®80) concentration		
1.6%	132.1±24.2	88.7±3.8
1.2%	233.3±39.6	110.3±9.1
1%	248.6±15.8	80.8±1.2
0.8%	251.9±35.3	73.9±3.9
0.6%	248.5±16.6	75.4±1.5
0.4%	134.1±32.1	65.5±0.8
0.2%	121.6±21.8	54.8±2.9

[a]Standard deviation



(a) Chitosan microsphere without drug



(b) Chitosan microsphere with drug

Fig.1. Scanning electron micrographs of chitosan microsphere

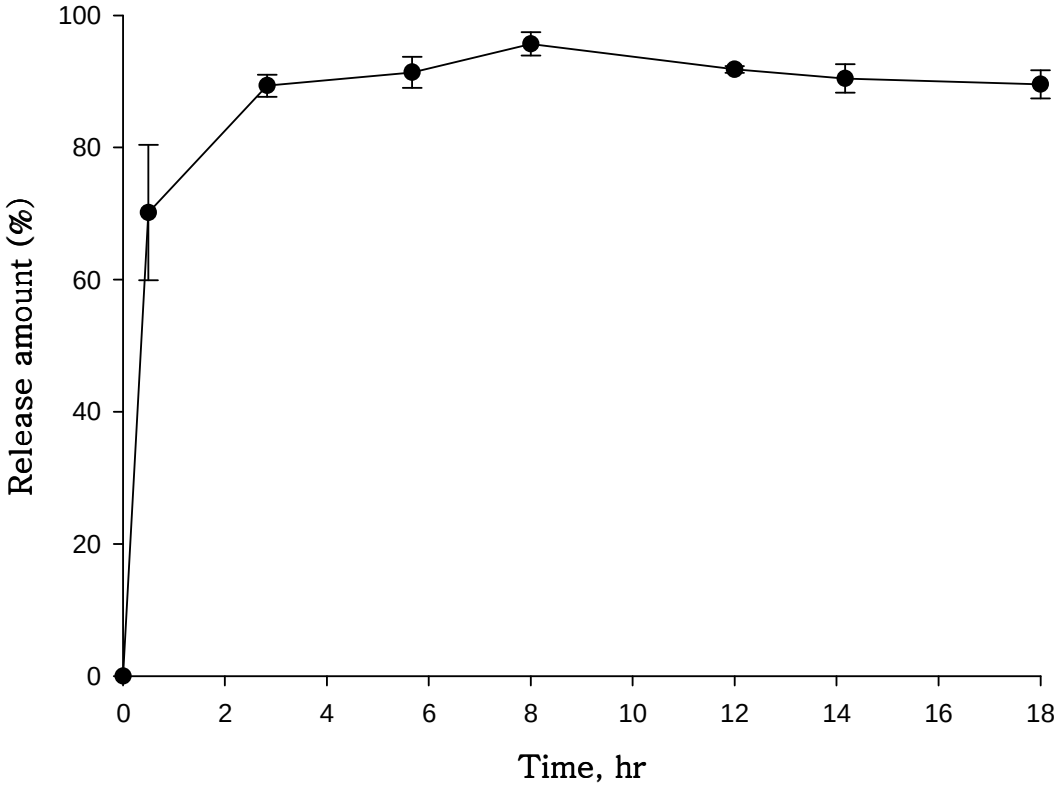


Fig.2. In vitro release of OTC from chitosan microsphere at pH 2.7 (n=3)

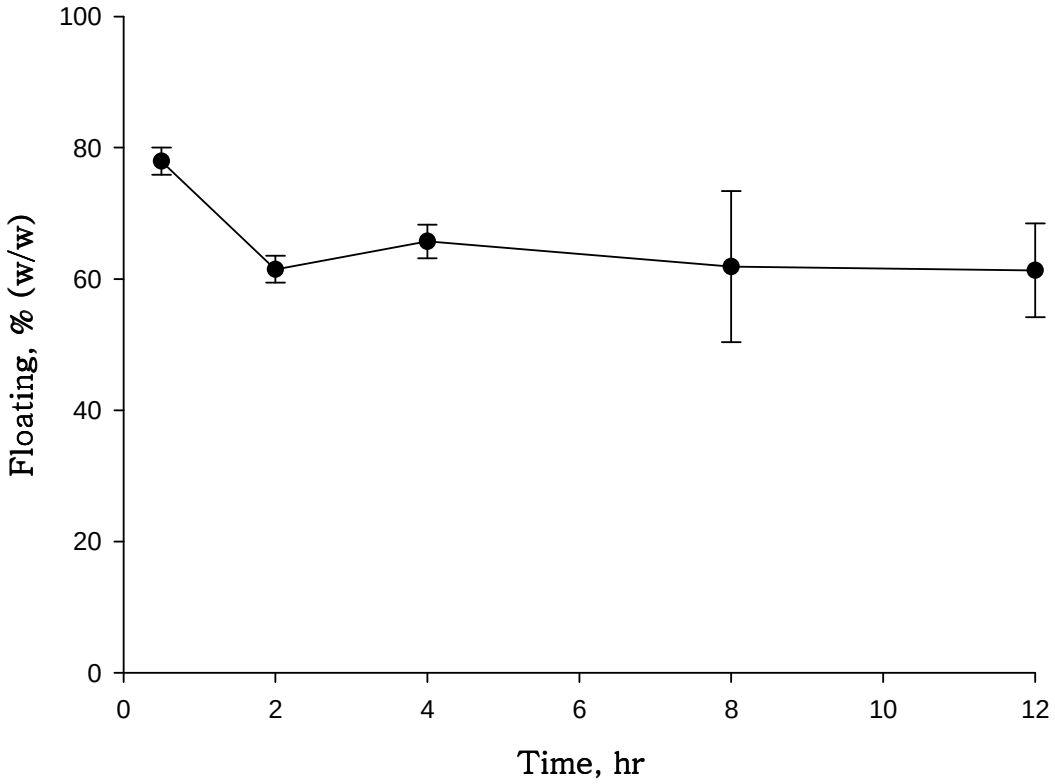


Fig.3. Floating behavior of chitosan microsphere on freshwater