Chitosan hydrogel microspheres: preparation, characterization, design of experiment and release studies

I. Lacík,[1]* S. Bubeníková,[2] P. Alexy,[2] L. Vodná,[2] D. Chorvát Jr.,[3] D. Bakoš[2]

[1] Polymer Institute, Slovak Academy of Sciences, Bratislava, Slovakia (igor.lacik@savba.sk)
[2] Institute of Polymer Materials, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, Slovakia
[3] International Laser Center, Bratislava, Slovakia

Introduction

Chitosan (CHIT) has become a traditional polysaccharide for formulations of materials (beads, capsules, spheres, membranes, sponges) where its advantageous characteristics are utilized.[1] These include availability and cost but mainly biocompatibility, biodegradability, bioadhesion, antiviral, antifungal and antibacterial activities. In recent years, CHIT microspheres and microcapsules have been used for the controlled release of bioactive substances such as antibiotics, proteins, peptides, drugs and vaccines.[2]

To tailor CHIT microspheres for controlled drug delivery, complexation between oppositely charged molecules has gained a lot of attention.[3-5] The most-studied system is based on the ionotropic gelation of chitosan with tripolyphosphate (TPP), which is non-toxic multivalent anionic counterion to amine groups of chitosan. This binary system exhibits, however, poor mechanical strength, which can be a limiting factor for intended applications. To eliminate this drawback, high molecular weight counterions, i.e., polyanions, such as alginate[6] and dextran sulphate[3] are used to form the polyelectrolyte complex with CHIT thus improving mechanical properties and tailoring permeability, chemical stability and controlled release properties as well.

In our work, we focused on producing the CHIT microspheres aimed at controlled release of the antibiotics for wound healing as the alternative strategy to CHIT-based membranes already tested in clinics.[7] Microspheres appear to be advantageous in terms of both effective wound covering and easier manipulation. They have to be used in form of a hydrogel to avoid patient's discomfort if, for example, more typical dried CHIT microspheres were used, which would swell and cause pain after covering the wound. In our study, CHIT/TPP microspheres were reinforced by anionic chondroitin sulphate[8] (CHS) as another biocompatible, biodegradable and bioactive natural polymer. We optimized the chemistry of microspheres and process conditions employing the statistical Design of Experiment (DoE) method.[9] This allowed for identification of optimal formulation parameters for preparation of chitosan hydrogel microspheres with improved mechanical resistance and controlled structure. Ofloxacin (OF), the fluoroquinilone antibiotic, commonly used for treatment of a variety of bacterial diseases,[10] was tested as a model drug in the *in vitro* experiments.

Materials and methods

Materials. High viscosity chitosan (CHIT) of M_w ~ 800 kDa and degree of deacetylation 78% (Fluka), tripolyphosphate (TPP) (Acros Organics) and chondroitin-4-sulphate (CHS) (Merck) were used for microsphere preparation. Ofloxacin (OF) (Sigma) was used as encapsulated antibiotic. All other chemicals were of analytical grade.

Solution preparation. Chitosan solution was prepared by dissolving CHIT powder and NaCl in 0.5 wt% acetic acid solution (pH 2.2) under stirring at 40°C, followed by pH modification with 1 wt%

NaOH aqueous solution and filled with distilled water so that the final concentration of each chitosan solution was 1 wt%. Polyanion solutions were prepared by dissolving TPP or TPP and CHS in saline and in distilled water, respectively. Tables 1 and 2 provide the ranges of polymer concentration and solutions pH for both the DoE experiments and experiments with ofloxacin loaded microspheres.

Microsphere preparation. Microspheres were prepared using various encapsulation procedures and processes. The procedures involved dropping CHIT (or CHIT with OF) solution to either TPP solution or solution of TPP and CHS, when the former CHIT/TPP microspheres were or were not coated with CHS. Regarding processes, two different processes were employed, namely the continual and discontinual ones. In continual process, CHIT (or CHIT/OF) solution was dropped trough a concentric nozzle producing droplets by air-striping of size of around 1 mm into the TPP solution continuously flowing in the multi-loop reactor[11] where the CHIT/TPP microspheres are formed. The reaction time of 40s was set by the flow rate of the TPP solution 60 ml/min and 4 loops. Throughout the discontinual process, the droplets of CHIT or the CHIT/OF solution were collected in 20 ml of anionic solution with collection time 5s and reaction time 35s. The flow rate of CHIT solution was around 0.8 ml/min. After the reaction, all types of microspheres were separated, washed, collected and stored at 4°C in saline solution or in distilled water containing 100 ppm of NaN_3 depending on the selected preparation conditions.

	Conc. (wt%)	pH	Reaction time (s)
CHIT	1	3 - 5.25	35
TPP	0.3 - 1	6 - 8	
CHS	0.3 - 1	6 - 8	
NaCl	0.9 - 1.2	-	

Table 1 Preparation conditions for DoE

	Conc. (wt%)	pH	Reaction time (s)
CHIT	1	5	40 - 300
OF	2	-	
TPP	0.5	7	
CHS	0.3 - 1	7	
NaCl	0 - 0.9	-	

Table 2 Preparation conditions for ofloxacin loaded microspheres

Optical microscopy. The microsphere diameter and membrane thickness were measured by optical microscopy (Kapa 2000, Kvant, s.r.o. Bratislava, Slovak Republic) using CCD camera (CC-63KW1P, Mintron Malaysia), by digital imaging using Image Forge 1.1 software. Microsphere diameter ranged between 600-900 μm. Typically, the microspherese were of a core-shell structure.

*Mechanical resistance.*The mechanical resistance of microspheres was measured using a Texture Analyser (TA-XT2i, Stable Micro Systems, Godalming, England) as the mean force necessary to break one microsphere. An applied compression speed of 0.5 $mm.s^{-1}$ was used. The data from the bursting force at the rupture point and the bursting force in 80, 90% distance were collected and recorded from the force-deformation curves. The bursting force values are presented as the mean of minimum 25 measurements.

Ofloxacin encapsulation efficiency and release kinetics. Receiving anionic solution above microspheres and two 5 ml washings were analyzed by UV/VIS spectrometry for ofloxacin released during encapsulation procedure. Cecil CE 7250 UV/VIS spectrophotometer was employed at 293 nm, which is the characteristic wavelength for OF. The encapsulation efficiency was calculated as the ratio (%) of encapsulated amount of OF and the theoretical amount of OF. The OF release kinetics was determined by UV/VIS spectrometry from the solution above 1 ml of microspheres stored in 10 ml of 0.9 wt% NaCl or distilled water at 37°C. The kinetics was followed during 28 days.

Results and Discussion

Design of experiment (DoE). We decided to employ the DoE scheme in spite of abundant information on CHIT microspheres. There are many parameters influencing the final CHIT microsphere properties which cannot be rigorously tested if not considering the interactions of various variables.

DoE requires to identify the boundary conditions. In the preliminary work, different types of microspheres were prepared based on the literature data to determine which variables are important in what range:

1. Chitosan of the molecular weight used in our work is soluble only under acetic conditions and above pH 6 it precipitates. Therefore, the pH of the CHIT solution was set to be in the range from 3 to 6.
2. Stable microspheres prepared solely from CHIT and CHS cannot be prepared via polyelectrolyte complexation between CHIT and CHS. Therefore, an addition of TPP is strongly required which via ionotropic gelation with CHIT forms a kind of scaffold for polyelectrolyte complexation between CHIT and CHS.
3. The pH of the polyanion solution was set to be in the range from 6 to 8 to cover the physiological range. Using acidic conditions for microsphere formation (p*H* = 3) is also possible but microspheres disintegrated within a few days.
4. Additional variable was ionic strength of receiving anionic solution. This parameter was tested based on findings of Bartkowiak that the ionic strength (and the p*H*) of the solution utilized during the capsule formation strongly influence the structure of the alginate-oligochitosan membrane.[12]

Consequently, the DoE scheme tested the effects of chondroitin sulphate, and tripolyphosphate concentrations, ionic strength, pH of both the polyanion solution (PA) and the chitosan solution (PC) on microsphere properties.[9] The range of each variable is shown in the Table 1 and the experimental design involves five factors on five levels:

X_1 = TPP/CHS (1)
X_2 = (TPP+CHS)/NaCl (2)
X_3 = NaCl /H_2O (3)
X_4 = p*H* PA (4)
X_5 = p*H* PC (5)

A design matrix consisting of 32 experimental runs was constructed. Microsphere diameter, membrane thickness, centricity, ovality and mechanical resistance were evaluated in the experiments using the STATIS software.[9] The system of regression equations is the output of the DoE, where the regression and interaction coefficients measure the response associated with each combination of factor and level, *i.e.*, the significance of the individual factors and their mutual interactions. The structure of microspheres in terms of membrane thickness is mainly dependent on the ionic strength and the pH of both the PA and the PC solution, when higher pH of PC solution leads to a thicker membrane. Microspheres exhibited minimum ovality (bellow 10 μm) and centricity above 95%. The mechanical resistance depends, except for factor 2, on all of the studied factors. CHS improved the mechanical resistance of microspheres with the maximum values of bursting force of around 3 g/microsphere.

Ofloxacin release. The different types of chitosan microspheres in terms of composition and preparation conditions were compared with respect to the release of ofloxacin. The encapsulation efficiency varied from 45 to more then 90 % depending on the microsphere preparation conditions. CHS effectively decreases the rate of OF release, which is deemed as beneficial feature in case of

controlling the drug release depending on the severity of infection. Additionally, the time of exposure of the CHIT/TPP microspheres to CHS solution is inversely proportional to rate of ofloxacin release.

It should also be noted that CHIT hydrogel microspeheres involving TPP ionotropic gelling step are highly sensitive to reaction time, which must be controlled in the range of tens of seconds. Therefore, the preparation of therapeutic amount of OF-loaded hydrogel microspheres requires utilization of the multiloop reactor.

Conclusions

This contribution deals with optimization of chitosan hydrogel microspheres based on chitosan complexation via electrostatic interactions with tripolyphosphate and chondroitin sulfate using various preparation and process conditions. To understand the microsphere formation, the design-of-experiment scheme was employed. The preliminary experiments of ofloxacin loading microspheres have shown its potential in controlling the release kinetics based on preparation conditions. As the ultimate aim of this work is to prepare biodegradable microspheres with controllably released antibiotics, the follow-up work will be devoted to the tests simulating the *in vivo* conditions.

Acknowledgements

This work was supported by Science and Technology Assistance Agency under the contract numbers APVV-51-033205 and APVT-20-015904.

References

[1]Kumar (2000*) A review of chitin and chitosan applications.* Reactive Funct Polym 46 1-27.

[2]Agnihotri et al. (2004) *Recent advances on chitosan-based micro- and nanoparticles in drug delivery.* J. Control. Release 100 5-28.

[3]Lin et al. (2005) *pH-sensitive polyelectrolyte complex gel microspheres composed of chitosan/sodium tripolyphosphate/dextran sulfate: swelling kinetics and drug delivery properties.* Colloids and Surfaces B: Biointerfaces 44 143-151.

[4]Ko (2002) *Preparation and characterization of chitosan microparticles intended for controlled drug delivery* Int. J. Pharm. 249 165-174.

[5]Akbuga et al. (1994) *Preparaton and evaluation of cross-linked chitosan microspheres containing furosemide.* Int. J. Pharm. 111 217-222.

[6]Bartkowiak. (2001) *Effect of the ionic strength on properties of binary alginate/oligochitosan microcapsules.* Colloid Surf A: Physicochem. Engin Aspect 204 117-124.

[7] Kukolíková et al. (2006) *Optimization of the properties of chitosan lactate/hyaluronan film*. J. Appl. Polym. Sci. 100 (2) 1413-1419.

[8]Laabs et al. (2005) *Chondroitin sulfate proteoglycans in neural development and regeneration*. Curr. Opin. Neurobiol 15 116–120.

[9]Alexy (1998) *STATIS program for planning and evaluation of experiments.* Department of Plastics and Rubber, Faculty of Chemical and Food Technology, STU, Bratislava, Teaching texts.

[10]Fresta et al. (2002) *Combining Molecular Modeling with Experimental Methodologies: Mechanism of Membrane Permeation and Accumulation of Ofloxacin.* Bioorg. Med. Chem 10 3871–3889.

[11]Anilkulmar et al (2001*) A novel reactor for making uniform capsules*. J. Microencapsulation, 18, 479.

[12]Bartkowiak et al (1999) *Alginate-Oligochitosan Microcapsules: A Mechanistic Study Relating Membrane and Capsule Properties to Reaction Conditions.* Chem. Mat. 11 (9) 2486.