



# Production of chitosan microparticles cross-linked with genipin – Identification of factors influencing size and shape properties



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## ABSTRACT

Biopolymer-based microparticles find application in a wide range of biomedical uses. Among other applications, microparticles are being used as drug-delivery systems. Chitosan is among the group of biopolymers with larger dissemination. The water-in-oil (w/o) emulsion method is employed to produce microparticles of chitosan chemically cross-linked with genipin for drug release purposes. The mechanical and transport properties of microparticles are affected by size and shape, which are, on its turn, influenced by input factors. In this study we investigate the impact of the concentrations of biopolymer and cross-linker, as well as the influence of the stirring rate on size and morphology of microparticles. A full factorial design of experiments is used to find the significant factors, and image analysis techniques are exploited to characterize the particle size and shape distributions. The results demonstrate that the concentrations of biopolymer and cross-linker, and the stirring rate have a significant impact on size properties but no impact on shape metrics. The increase of biopolymer and cross-linker concentrations provokes an increase in microparticles size, and the increase of the stirring rate has the opposite effect.

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## 1. Introduction and motivation

The efficacy of many drugs is often limited by low solubility, high potency requirement, and/or poor stability. In addition, in many cases, only a small fraction of the administered dose reaches the target site, while the majority diffuses throughout the body [1,2]. The design of drug delivery systems capable of releasing therapeutic agents under a controlled regimen of concentration, duration of action, and specificity, so that the efficacy and compliance are optimized and the side effects minimized, is a challenging topic in Biomedical Engineering field. One of the most promising approaches developed over the recent years is based on the use of the so called *microparticle-based therapy systems* which employs devices, such as microspheres, to encapsulate the drug and release it at controlled rates for relatively long periods of time in the target site [3]. Such systems offer several advantages over conventional methods of administration: (i) the drug release profiles can be tailored to the needs of a specific application; (ii) the controlled release

systems provide additional protection to drugs, increasing the efficacy; and (iii) increase patient quality of life and compliance [1,4].

A number of materials have been investigated for producing microspheres to use as drug carriers. Biocompatible and biodegradable materials offer several advantages, since they allow to avoid complex and painful procedures for removing the drug depleted matrix. Chitosan (CH) is a degradable biopolymer with a set of properties that makes it suitable for controlled drug release applications ranging from hydrogel supports to microspheres [3,5–7]. From the chemical point of view, chitosan is a copolymer of glucosamine and N-acetylglucosamine, derived from chitin (for a complete reviews on chitosan properties and applications see [8–10]), that possesses unique features related to biodegradability, hydrophilicity, absence of toxicity and non-antigenic properties, anti-microbial activity, bioadherence and cell affinity as well as hemostatic potential [8,11]. This combination of properties allows to exploit the chitosan for producing microspheres to encapsulate drugs, such as antibiotics, steroids, antihypertensive, anticarcinogenic, and antidiabetic agents, diuretics, proteins, amino acids, peptides and vaccines [3,12,13], and subsequently functionalize those systems for drug delivery.

Chitosan microspheres can be obtained by promoting the ionic interaction of the amine groups of the polymer with multivalent anions provided by polyphosphates and sulphates, and is

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designated *physical cross-linking* [14,15]. Another production strategy involves the cross-linking reaction of the amine groups of chitosan with other groups provided by synthetic agents such as diisocyanate or epoxy compounds, and is called *chemical cross-linking* [3,12]. The cross-linker agents employed in this synthesis route are very often cytotoxic, which may impair the biocompatibility of the microspheres and constrain the range of applications. An approach to handle this limitation consists in using cross-linkers with low cytotoxicity but which, nevertheless, allow the formation of stable and biocompatible polymeric structures with mechanical properties that ensure its integrity [16–19]. Among the most widely used biocompatible cross-linkers satisfying those criteria is Genipin, a naturally occurring agent.

Chitosan cross-linked microspheres have been prepared by a number of physical processes, namely coacervation/precipitation [20], emulsion [15,21–23], sieving [24], spray-drying [25]. The emulsion methods require a sequence of washing steps, which makes them complex, especially for industrial exploitation. Methods based on atomization/coagulation processes have higher complexity in terms of the operations required, but are more attractive for industrial application. However, the costs of the equipment and its operation are much larger. For production at lab scale the emulsion-based methods are still competitive and requires lower investment. Among the emulsion-based methods there is also differences related with the phases involved; one of the most commonly used being the water in oil (w/o) method [15,26]. From our knowledge, an automated and simple method for producing microspheres for drug encapsulation remains to be identified and optimized.

Several studies demonstrate that particles produced employing the emulsion method have spherical shape [15]. In our experiments, we expect results in agreement with that postulate. However, as far as we know, the literature available is limited regarding the quantitative characterization of the shape of microparticles. Very often the particles are described as spherical and smooth in result of optical microscopy/SEM analysis techniques [27], but a measure of roundness/circularity is not assessed. The impact of particles shape on the parameters that govern the functional behavior of carriers is not well understood but a number of reports have emerged proving that shape can affect, for instance, the phagocytosis in *in vivo* systems, and consequently the drug release kinetics, see [28] among others. The impact of the shape has not been thoroughly studied due to difficulties in producing and measuring particles of different forms. Further, there is no evidence that even small variations in the shape cannot induce different behaviors of the carrier. The technique commonly used to determine the particle size distribution (PSD) is based on laser diffraction, which does not allow to characterize the shape. To overcome this problem we use a technique based on image analysis that is able to determine both the size and shape of the particles, and can be automated to provide accurate measures in realistic times, thus solving the problem of measuring the later variable.

Previous studies have shown that, for the w/o emulsion method, factors such as the polymer concentration, the cross-linker to polymer mass ratio, and the stirring speed of the emulsification step may impact the size and the shape of the microspheres [29,26,12,30]. The size and shape of microspheres, on its turn, may impact the pore structure, and, consequently, the transport properties of drug molecules within the matrix, which affects the rate of drug release [7,31]. Basic mechanistic knowledge predicts lower relative drug release rates as the size of microspheres increase, and prolonged drug release profiles for microspheres of broader PSD [1]. However, an aspect we consistently observed is that even at lab scale the effects of input variables on microparticles size and shape are not clear and deserve to be further investigated. This lack of knowledge can be extended to w/o emulsion method, where the

relation between factors to control in production phase and the microparticles size/shape is not established. In this study our goal is to identify the relations between factors to control in production step and the size/shape of chitosan cross-linked microspheres; we use a design of experiments (DoE) for such a purpose. Another innovative aspect of our work is the use of image analysis techniques for analyzing size and shape of biopolymer microparticles synthesized for controlled drug release applications.

## 2. Materials and methods

### 2.1. Materials

The chitosan used in the experiments (molecular weight  $\approx$  200 kDa and a degree of deacetylation of 87%, calculated from the carbon/nitrogen ratio by elemental analysis) was purchased from Sigma-Aldrich in powder form. Genipin, in form of crystal-like powder of reagent grade, was supplied by Challenge Bioproducts, Taiwan. Mineral oil (viscosity: 16.8 mm<sup>2</sup>/s at 40 °C, and density: 848.5 kg/m<sup>3</sup> at 20 °C) and Span80, an oil-soluble emulsifier, were supplied by Merck. All of the other reagents and solvents used in this work were of the highest purity commercially available.

### 2.2. Method of production of microparticles

Chitosan microparticles were prepared by w/o emulsion method followed by cross-linking using genipin. The preparation procedure is similar to those reported by Kawadkar [26] and Karnchanajindanun et al. [30] with minor modifications.

Chitosan is dissolved in aqueous acetic acid solution (0.5%, v/v), and the resulting solution is the water dispersed phase. A volume of 3 mL of this solution is then dispersed, drop by drop, into a 40 mL of mixture of mineral oil and Span80 (2%, w/v), which is the organic phase, and then homogenized with continuous stirring using a magnetic stirrer Selecta, Agimatic-N (of J.P. Selecta, S.A., Barcelona, Spain). The water to oil phase ratio is 1/13.3 (v/v). An emulsion of microparticles of chitosan is formed within the oil phase. Then, a volume of 0.5 mL of genipin 70% (v/v) alcoholic solution is added drop-wise to w/o emulsion, to obtain a solution with a ratio of genipin to chitosan of 1/6 (v/v). In all experiments the same volume of cross-linker solution was used, so that the viscosity of the emulsion remains constant. The cross-linking of microparticles is carried out during 6 h at 37 °C, under continuous appropriate stirring. Afterwards, the microparticles are separated from the continuous phase by decanting, then are submitted to washing with hexane and again decanted three times. Finally, the microparticles are dried at room temperature. The complete procedure is schematically presented in Fig. 1. The levels of the factors to control in the preparation procedure will be set in Section 2.5.

### 2.3. Analytical and qualitative characterization

The shape and surface of chitosan microparticles was examined via scanning electron microscopy (SEM). A small amount of microparticles previously dried were sprinkled onto a double adhesive fixed on aluminum stage. Fixed microparticles were then spattered with gold film, and observed in a JSM-5310 scanning microscope (of JEOL, Tokyo, Japan) operating at 15 kV.

Fourier Transform Infrared Spectroscopy (FTIR) was used to investigate the cross-linking reaction of chitosan amino groups with genipin. Microparticles used in the analysis are initially lyophilized (in a lyophilizer Snijders Scientific type 2040, Tilburg, Holland) and ground into powder form. Then, the powder is mixed with KBr (in a ratio 1:100) and pressed into a pellet. The FTIR spectra is recorded using a FTIR-4200 Fourier transform infrared spectrometer (of Jasco, Tokyo, Japan) operating at room temperature, in the

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