J Mater Sci: Mater Med (2006) 17: 371–377
DOI 10.1007/s10856-006-8240-z

# Starch-based microspheres produced by emulsion crosslinking with a potential media dependent responsive behavior to be used as drug delivery carriers

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Received: 14 April 2004 / Accepted: 13 July 2005
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**Abstract** This paper describes the development and characterization of starch microspheres for being used as drug delivery carriers in tissue engineering applications. The developed starch microspheres can be further loaded with specific growth factors and immobilized in scaffolds, or administrated separately with scaffolds. Furthermore and due to the processing conditions used, it is expected that these microspheres can be also used to encapsulate living cells. The aim of this study was to evaluate the efficacy of this methodology for further studies with biologically active agents or living cells. The starch microspheres were prepared using an emulsion crosslinking technique at room temperature to allow for the loading of biologically active agents. A preliminary study was performed to evaluate the incorporation of a model drug (nonsteroidal anti-inflammatory drug-NSAID) and investigate its release profile as function of changes in the medium parameters, such as ionic strength and pH. The developed starch-based drug delivery system has shown to be dependent on the ionic strength of the release medium. From preliminary results, the release seems to be pH-dependent due to the drug solubility. It was found that the developed microspheres and the respective processing route are appropriate for further studies. In fact, and based in the processing conditions and characterization, the developed system present a potential for the loading of different growth factors or even living cells on future studies with these systems for improving bone regeneration in tissue engineering, especially because the crosslinking reaction of the microspheres takes place at room temperature.

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## 1. Introduction

There are multiple clinical reasons to develop bone tissue-engineering alternatives in order to improve the quality of life of patients. There is a clear need of better scaffold materials and cell-loaded constructs that can be used in the regeneration/engineering of large orthopaedic defects. The main factors necessary to make a tissue engineering approach successful are, in a very simplistic way [1]: (i) scaffolds (ii) cells, and (iii) growth factors. As a simple description, it is necessary a scaffold as a support on which cells (after their isolation and expansion) can be seeded and then will proliferate and differentiate into the aimed phenotype. In addition, growth factors are required to improve cell differentiation and proliferation and then achieve tissue regeneration. The controlled release concepts can be inherent and be a potential assistant in the three main factors [2]. Referred to above, the 'traditional' drug delivery approach can be further applied in tissue engineering to encapsulate living cells for incorporation within the scaffolds. In turn, scaffolds can be designed as 'traditional' drug delivery carriers to control a site-and time-specific release profile of biologically active agents and also to protect the growth factor.

In our point of view, biodegradable microspheres that can be used as controlled release systems are a key issue for the development of an optimal strategy for tissue engineering applications. The basic idea to try to accomplish in tissue engineering applications is that biodegradable microspheres can be either simply injected at the site of regeneration

together with the scaffolds or coupled/immobilized into or in the surface of the porous scaffold. Nevertheless, the developed starch microspheres can be used as 'traditional' drug delivery systems to local delivery of bioactive agents. Starch-based systems is very promising for several biomedical applications [3–16], including drug delivery carriers [17–20]. However, up to date there is no information that starch microspheres have been used for this propose. On the other hand, biodegradable starch-based microspheres have been widely investigated and even used for a long time as 'traditional' drug delivery systems [21–23].

The ability of polysaccharides to form a network structure (gel), even at very low concentrations, constitutes one of their most important functional properties. The formation of a 3D network structure (gelation) offers an effective means of increasing the chemical stability and mechanical properties of the system [24, 25]. A wide range of modification mechanisms that can be applied to starches is known [24, 25]. These include self-association (induced by changes in pH, ionic strength or physical and thermal means), complexation with salts and covalent crosslinking as used in the present study. Furthermore, starch is appealing to the area of drug delivery as it allows to produce systems of a low toxicity that are biodegradable and very stable [26]. Obviously, it is important to emphasize the high availability of starch and remember its renewable origin. In fact, starch is the most abundant reserve polysaccharide of the plant kingdom. Therefore, the cost-effectiveness of starch-based products is another important attractive for the use of starch in the drug delivery field as well as in other biomedical applications.

In particular, starch microspheres have been widely investigated for different drug delivery applications [21–23, 26, 27]. For instances, Illum *et al*. [26] reported on bioadhesive microspheres that could not be cleared easily from the nasal cavity. The half-life of clearance for starch microspheres was in the order of 240 min as compared to 15 min for the control formulations. If gentamycin was administrated in combination with starch microspheres, a significant increase in bioavailability was obtained [26]. Another example is the magnetic starch microspheres developed by Fahlvik *et al.* [23, 28] for parenteral administration of magnetic iron oxides to enhance contrast in magnetic resonance imaging. This could be an interesting processing route to be used in the future to try to develop magnetic-responsive controlled systems for delivery of biological active substances.

The use of a particulate embolic agent combined with regional chemotherapy now in clinical use in the treatment liver cancer has been widely investigated [21]. In this treatment, degradable starch microspheres (DSM) play an important role. Furthermore, Bjork *et al*. [22, 29] reported the use of these microspheres as nasal delivery systems for drugs such as insulin.

Concerning the oral administration is important to note that native starch is almost completely broken down after oral ingestion [30, 31]. This is done by pancreatic enzymes that lead to the subsequent absorption from the small intestine into the systemic circulation. A certain proportion of starch, called resistant starch, escapes digestion in the small intestine and undergoes fermentation by bacteria in the colon [32, 33]. However, the enzymatic degradation taking place in the stomach needs to be controlled to achieve an adequate delivery of the bioactive agents. With this aim, Larionova *et al.* [34] developed crosslinked starch-protein microcapsules containing proteinase inhibitor in order to allow for the oral administration of the proteic or peptide drug. The protective effect of microcapsules with aprotinin for bovine serum albumin was revealed *in vitro* [34].

As it is possible to conclude from the previous observations, there have been a number of applications of starch microspheres in the drug delivery field. The present paper describes the development and characterization of starch microspheres for drug delivery applications. The microspheres were prepared using an emulsion crosslinking technique. A preliminary study was performed to evaluate the incorporation of a model drug (nonsteroidal anti-inflammatory drug-NSAID) and investigate its release profile in function of changes in the medium parameters. The idea is to develop systems that might be used in tissue engineering applications, where the starch microspheres can be either simply injected at the site of regeneration simultaneously with the scaffold or coupled with/into the porous scaffold. Furthermore and due to the processing conditions used, it is expected that these microspheres can be also used to encapsulate living cells.

## 2. Materials and methods

Paselli (II) from Avebe (The Netherlands) which is a modified potato starch was used in this study. As it is well known, native starch is not soluble in water below 80°C. This can be a clear disadvantage in some reactions. To overcome this disadvantage, native starch is partially hydrolyzed being modified into several modified starches, such as Paselli (II), which is a water-soluble starch. Trisodium trimetaphosphate (TSTP) was used as starch crosslinking agent Span 80 and Tween 80 were used as hydrophobic and hydrophilic surfactants, respectively. All the reagents were purchased to Fluka and used as received.

The drug used in this study was meclofenamic sodium salt (MS) which is a non-steroidal anti-inflammatory drug (NSAID) mainly used for the treatment of arthritic inflammations (osteo and rheumatoid). The drug was approved by the United States' Food and Drug Administration (FDA) for the treatment of mild-to-moderate pain in August 1988 [35].

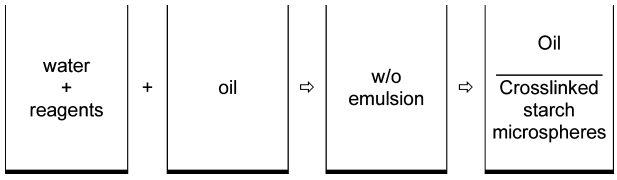


**Fig. 1** Schematic representation of single emulsion crosslinking technique.

Previously this drug has been incorporated into porous starch-based drug delivery carriers [17].

For the production of starch microspheres a single emulsion crosslinking technique was used. A scheme of the procedure is presented in Fig. 1. Briefly, the water-soluble starch (25% wt) and the crosslinker agent (5% wt) are dissolved in the water phase under constant stirring at room temperature in a glass beaker. A mechanical stirrer coupled to the assembly controls the stirring.

The water-phase (w) and the oilic-phase (o) are mixed (1:1), using (or not) a hydrophobic surfactant (5% wt), depending on the selection of reaction conditions. After this step, a w/o emulsion is obtained. The crosslinking reaction is started by raising the pH to a 12-13 value with a NaOH solution (2% wt) to activate the crosslinking agent, maintaining the stirring at constant rate. Samples were collected to control the crosslinking reaction at different periods. When the collected sample is crosslinked, the reaction is stopped by means of neutralizing with HCl solution. In order to obtain the microspheres, the phases are separated at the end of the reaction with ethanol. The reaction product is then decanted or centrifuged (depending on the microspheres size) and washed, repeating this step several times until total clearance of the oilic-phase. (Note: all the weight percentages indicated are relative to the water-phase).

In order to load the starch microspheres with the biologically active substance (2.5% wt), a double emulsion crosslinking technique is used as represented in Figure 2. The main difference on the processing route is that it is first obtained a drug/w emulsion by adding a hydrophilic surfactant. All the sequence of procedure is similar to the one for single emulsion technique and for that reason it will not be repeated herein.

In the particular case of starch microspheres, the stirring and the presence of surfactant was varied to study its effect on particle size. Table 1 summarizes the conditions for each crosslinking reaction and the respective sample designation which will be used for further discussion in this paper.

**Table 1** Designation of starch microspheres according the crosslinking reaction conditions.

| Designation | Surfactant | Stirring Rate (rpm) |
|---|---|---|
| S1 | Yes | 1200 |
| S2 | Yes | 700 |
| S3 | No | 700 |

The developed starch-based microspheres were morphologically characterized by Scanning Electron Microscopy (SEM), in a Leica Cambridge S360. All the samples were previously gold coated. The SEM analysis allowed for the characterization of the morphology of the developed microspheres, to evaluate qualitatively the particle size and particle size distribution. Furthermore, it was used to identify any morphological changes after release tests. Optical microscopy was also used to follow the morphology during the crosslinking reaction using an Eclipse E400 optical Nikon microscope and images were recorded with a Nikon FDX-35 camera coupled to the optical microscope.

The particle size and particle size distribution were accessed in a Coulter LS 230 light scattering particle size analyzer by saturation in a dispersion of water.

The X-ray diffraction (XRD) spectra were acquired in a Philips X'Pert MPD diffractometer in order to characterized structurally the different particles developed as well as the main raw materials used.

*In vitro* drug release tests were carried out for starch drug-loaded microspheres at physiological temperature ($T = 37 \pm 1^{\circ}C$). Solutions with different salt concentrations [NaCl – 0 M, 0.154 M (physiological value) and 1 M] were used in order to increase ionic strength of the release medium and study this medium parameter in the release profile. Starch-loaded particles were placed in containers carefully sealed with test solution for periods up to 14 days. At different time periods, solution aliquots of 5 ml were colleted, and the same initial volume was adjusted with new fresh solution. The drug release was measured by UV spectrophotometry in a Shimadzu UV-1603 spectrophotometer taking the original test solution as reference.

## 3. Results and discussion

By optical microscopy it was possible to follow the crosslinking reaction. As shown in Fig. 3.a after 2 h of crosslinking

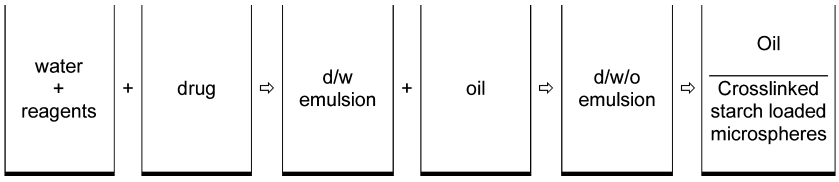


**Fig. 2** Schematic representation of double emulsion crosslinking technique.

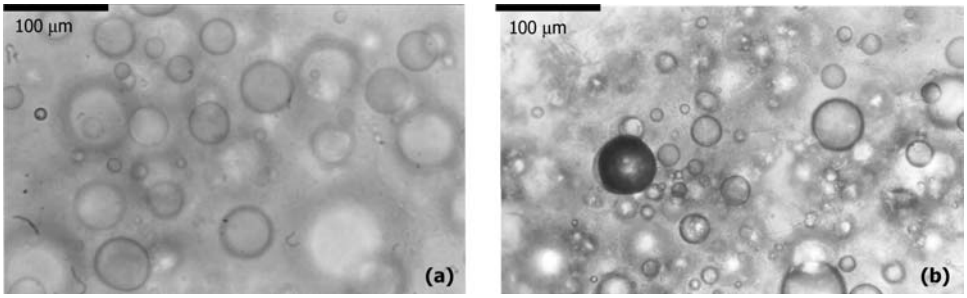


**Fig. 3** Optical micrographs showing the evolution of emulsion crosslinking reaction after (a) 2 h and (b) 6h.

reaction the starch microspheres were not totally formed. After 6h of reaction time, the starch microspheres shown to be morphologically stable and crosslinked. This reaction time was selected based in the study of the reaction progress at several time periods. The 6h reaction period was the optimal time in terms of crosslinking and morphological stability of the starch microspheres. This value for reaction time was set and used in all reactions based in optical microscopy observations and following the crosslinking reaction by collecting several samples at several periods performing dissolution tests that shown that at this reaction time the starch particles were stable.

The morphology of the developed starch microspheres was accessed by SEM and it is shown in Fig. 4. It is possible to observe that the developed processing route allowed obtaining dense and approximately spherical microspheres.

By light scattering analysis it was possible to determine the particle size and particle size distribution as it is shown in Fig. 5. The emulsion crosslinking technique allowed for the controlled production of microspheres with a high array of mean diameters ranging from 3 to 540 $\mu$m by changing the reaction parameters. By using this characterization technique, it was possible to evaluate the effect of the different processing conditions studied, namely the influence of surfactant and stirring rate, in the microspheres size. It can be seen that the influence of surfactant is of major importance in the control of particle size since it was possible, with the use of surfactant, to decrease the microspheres mean diameter from 500 $\mu$m (S3) to 30 $\mu$m (S2). This is due to the decrease of interfacial energy between the polymeric phase and the crosslinking medium by the surfactant presence, allowing the stabilization of the pre-formed microspheres to maintain their size until completion of the crosslinking reaction.

The stirring rate also allowed a significant decrease in particle size by decreasing the mean diameter from 30 $\mu$m (S2) to 3 $\mu$m (S1). This parameter was also very effective since it is known that is rather difficult to decrease particle size below a certain small diameter due to the pre-formed

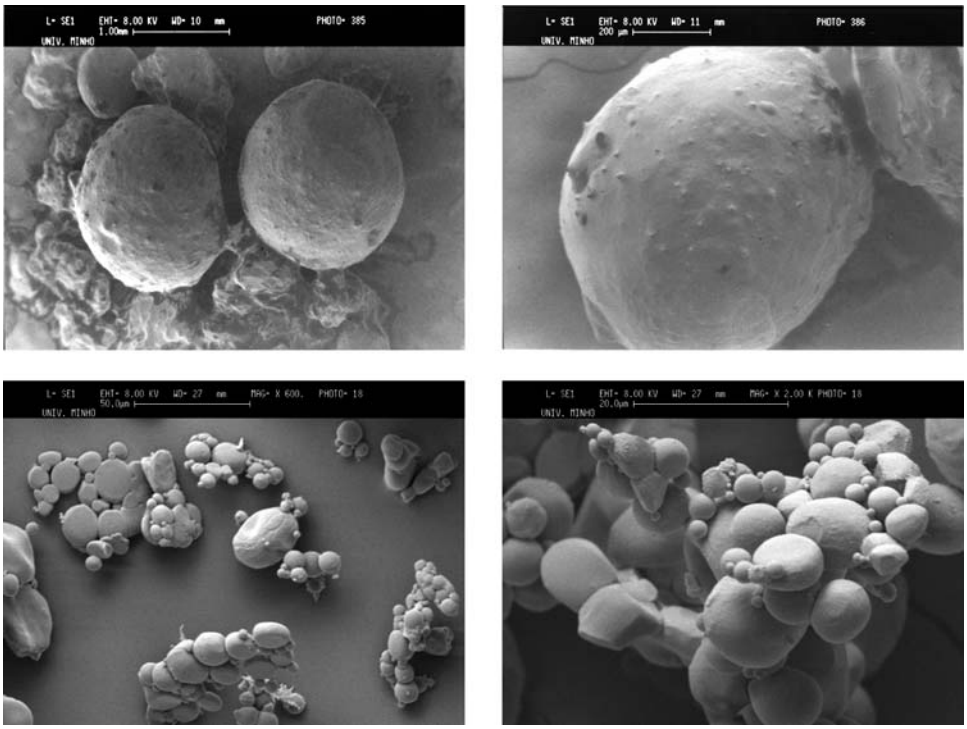

**Fig. 4** SEM micrographs showing the starch-microspheres morphology. (Please note the respective scale bars).

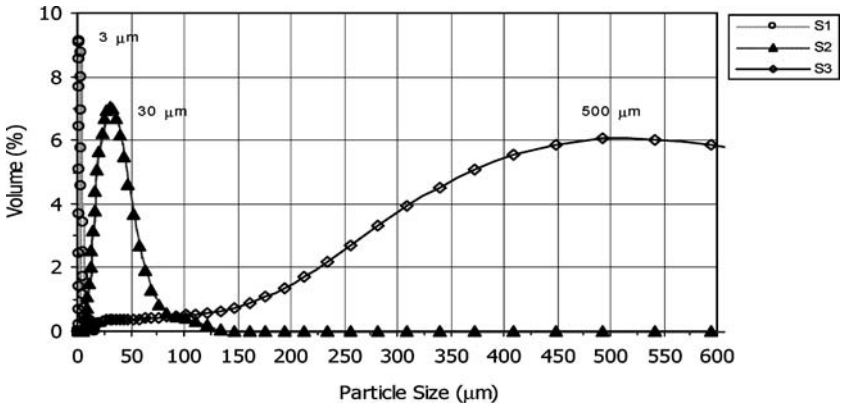


**Fig. 5** Particle size and distribution of starch microspheres as function of different reaction conditions (S1 – surf., ↑ rpm; S2 – surf., ↓ rpm; S3 – No surf., ↓ rpm). Please see Table 1 for more details.

microspheres stabilization. The influence of both the stirring rate and the presence and concentration of surfactant was already studied to have influence in the microparticles mean diameter [36].

X-ray diffraction was used to accessed the structural characterization of the developed starch microspheres and evaluate the influence of the processing route in the particles final composition. As it is possible to see in the XRD diffractograms shown in Fig. 6, there was an incorporation of sodium phosphate in the developed particles due to the selected processing route. This fact can be related to the presence of residual crosslinking agent.

*In vitro* release tests were carried out for starch microspheres with higher diameter (500 $\mu$m) in order to minimize sampling problems, since the aim of this study was to evaluate the influence of ionic strength and pH of release medium on the drug release. Nevertheless, it is expected that smaller microspheres show a similar release pattern with increased initial drug release due to higher surface area that leads to a higher water uptake. In initial release periods, the water uptake is the controlling mechanism since starch microspheres have a hydrophilic character.

From previous experiences it was concluded that the solubility of MS decreases strongly with decreasing pH and for this reason the particles were not neutralized prior to release tests. The pH of the release medium is defined from the NaOH concentration in the particles and solution, being $9.0 \pm 0.2$. An extra small amount of NaOH was added to the release medium in two release conditions in order to increase the initial pH medium value (up to $10 \pm 0.2$), facilitating the initial drug solubilization. For initial release periods, this increase in the pH value seemed to have no significant effect since, the release is very fast in the first 2 hours (Fig. 7) for all the studied mediums. Nevertheless, and comparing the mediums with the same salt concentration for higher release periods, the increase in the pH increments in some extent the drug release. One can concluded that the lower pH value ($\text{pH} = 9.0 \pm 0.2$) is not compromising the MS dissolution being high enough to dissolve all the MS released. Having in consideration the lower MS solubility at lower pHs, it is expected that, in an *in vivo* application, the initial burst release will be decreased due to the physiological pH value. Further studies with significantly different pH values are needed in order to be possible to undoutebouly classify theses systems as pH-dependent.

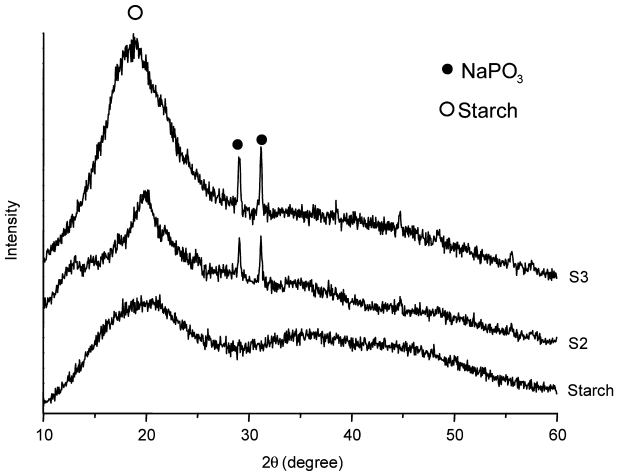


**Fig. 6** XRD diffractograms of starch microspheres obtained by emulsion crosslinking technique. Please note that (•) correspond to sodium phosphate.

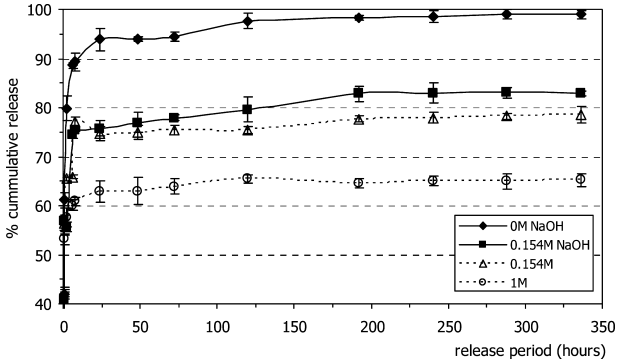


**Fig. 7** MS release profile of starch loaded microspheres as function of release medium.

It was possible to confirm that the release of the drug shown to be strongly dependent on salt concentration as it can be observed in the data plotted in Fig. 7 for different salt concentrations mediums. This can be attributed to the dependence of drug solubility and swelling behavior of the starch particles on the medium ionic strength. These parameters will control the MS release being possible to classify the developed starch-MS systems as responsive drug delivery systems in terms of ionic strength-dependence. This behavior has obvious advantages in *in-vivo* applications to maintain the balance of salt in cases for instances of perspiration. These systems can respond to a decrease or increase in the salt concentration releasing the appropriated bioactive agent previously incorporated to reestablish the equilibrium. In terms of bone tissue engineering, the equilibrium of salt concentration is of major importance for instances to maintain cells viability.

## 4. Conclusions

The single emulsion crosslinking technique allowed for the production of spherical starch microspheres with a high array of mean diameters ranging from 3 to 540 $\mu$m with a narrow size distribution for smaller diameters. This was achieved by varying the conditions of crosslinking reaction being demonstrated that the particles size can be controlled by means of changing the processing conditions. Concerning the initial release of drug from the starch microspheres it was possible to observe that the delivery is very fast in the first 2 hours and independent of medium conditions. For higher release periods, the increase in the pH increases in some extent the drug release, independently of salt concentration. Concerning the influence of medium ionic strength, it was demonstrated that the drug release was strongly dependent on salt concentration, being possible to classify this system as ionic strength dependent.

**Acknowledgements** Portuguese Foundation for Science and Technology (PhD Grant to P.B. Malafaya, SFRH/BD/11155/2002 under the POCTI Program). This work was partially supported by FCT Foundation for Science and Technology, through funds from the POCTI and/or FEDER programmes.

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