Preparation of agarose based microcapsules by emulsion and microdropplet generator techniques.

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Introduction

Agarose is a natural polymer extracted from seaweed (Craige J. S. et al. 1990) composed of alternating units of β-D-galactopyranosyl and 3,6-anhydro-α-L-galactopyranosyl units. It exhibits a temperature-sensitive water solubility and it provides a thermoreversible gels. Agarose is insoluble in cold water but dissolves to give random coils in boiling water. After cooling at room temperature, the aqueous solution forms stable and firm gels that do not melt below 85° C. The gel network of agarose contains double helices stabilized by the presence of water molecules bound inside the double helical cavity.
Agarose gel is widely used in various fields of food industry as stabilising and gelling additives. A traditional sector of application of agarose is the confectionery industry, where it is used as a jellifying agent for jellies and aerated candies. It also became best knows as a culture medium for the growth of microrganisms such as bacteria and fungi. Recently, agarose has found application also in pharmaceutical products as thickener and suspending agent, as laxatives and therapeutic agents in the treatment of malfunctions of the digestive tract, as a retarding agent and as carrier in formulations for controlled release of drugs. Futhermore, agarose gel is employed in various fields of biomedical research, particularly in tissue culture systems because it permits growing cells and tissues in a three-dimensional suspension. Sheets of agarose gels are frequently used in molecular biology for the separation of proteins through electrophoresis and in chromatography through exclusion of sizes.
The biocompatibility and the lack of ionic groups, make the agarose a suitable polysaccharide for the entrapment of mammalian cell. Spherical beads of agarose offer an optimal envoirement for the proliferation and the differentation of the cell This has been successfully used for cell encapsulation over the last couple of decades (Iwata H. et al. 1986) (Tun T. et al. 1996) (Gu Y.J.et al. 2000) but in the literature there is very little informations and only few articles describe the procedures for the encapsulation (Nilsson K. et al. 1987).
A simply method to prepare agarose particles has been described by various authors (Mu Y. et al. 2005) and consisting of dispersion of warm agarose solution in oil by stirring to produce an emulsion, then cooling to produce solid particles. The limits of this method are the not heterogeneous size distribution of the resultant capsules.

Materials and methods

Agarose for routine was purchased by Sigma-Aldrich (Germany), isopropyl myrystate used as an oil phase was purchased by Fluka; Span S 80 used as surfactant was purchased by Eigenmann and Veronelli (Italy). Other solvents were of reagent grade and were used without further purification. An appropriate amount of agarose powder (0.8-2% w/v) was suspended in an aqueous phase and heated at 100°C until a clear solution was obtained. Once the agarose was completely dissolved, the temperature was decreased to 75°C. At the same time, 50 ml of oil solution with surfactant (1-1.5% w/v) was heated to 75°C. 5ml of the agarose solution was dripped on hot oil solution and the mixture was emulsified by an impeller at different speed (500-700 rpm) for 2 minutes. The resulting emulsion was cooled by ice-cold bath and then left stirring for 5 minutes to convert the aqueous droplets of the agarose solution into agarose hydrogel particles. The agarose hydrogel particles were formed when the temperature of the emulsion was reduced below the gelation temperature of the agarose solution (approximately 36°C). Then, stirring was stopped and the suspension was left on

ice-cold bath for 5 minutes and the microparticles recovered by filtration. The morphological and dimensional analysis of the produced microparticles was evaluated by stereoscopic microscopy .

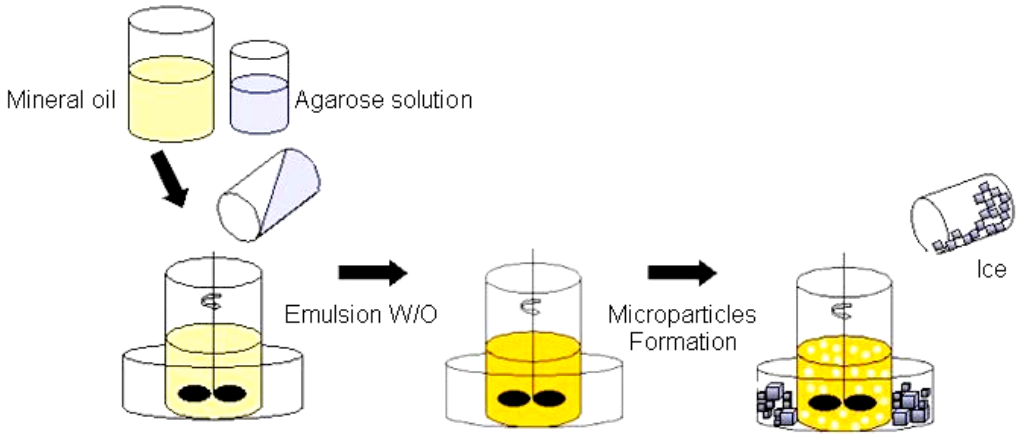

Fig. 1. Schematic rappresentation of the water in oil (w/o) emulsion method used for the production of agarose based microcapsules

Results and discussion

The aim of this study is to develop agarose microparticles by a water in oil (w/o) emulsion method (see the general production scheme in Fig. 1) with a narrow size distribution and to investigate the influence of the various manufacturing parameters such as agarose concentration, stabiliser concentration and impeller speed on microparticles morphology, size and size distribution.

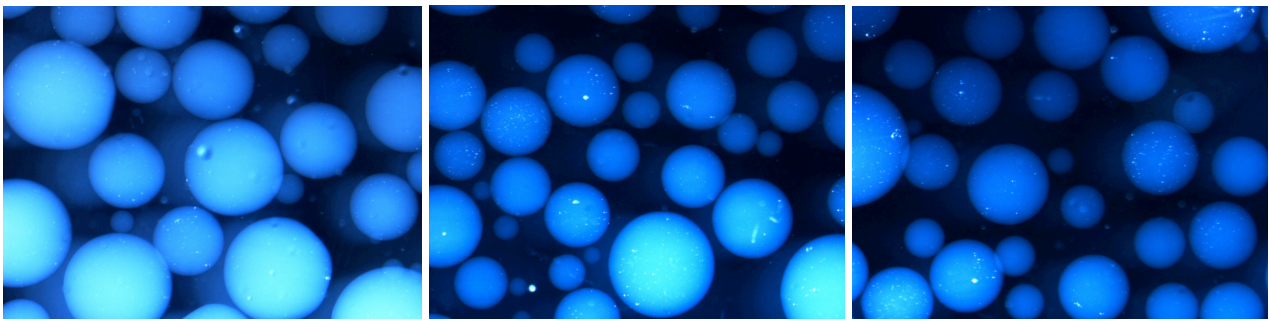

Fig. 2. Effect of the strirring rate on the morphological characteristics of the agarose microparticles. Stereomicrographs of agarose microcapsules manufactured by w/o emulsion using as continuous phase isopropyl myristate. Samples were prepared at 500 rpm (left panel), 600 (central panel) and 700 (rigth panel).

In order to evaluate the influence of the manufacturing parameters on the resulting agarose based microcapsules, one manufacturing parameter was changed keeping all others constant (stirring rate, agarose concentration and surfactant concentration). When the stirring rate was increased, a reduction of the mean diameter and lower polydispersity was observed through stronger shear forces and increased turbulence. A constant diameter reduction was observed passing from 500 to 700 rpm (from 416±38 µm to 237±28 µm) (Fig. 2, Tables 1 and 2).
The increasing of the surfactant concentration from 1% to 1.5%, produced a reduction of the microparticle size. At high surfactant concentration, the viscosity of the continuous phase will also increase, amplifying for a given stirring rate, the shear forces acting upon the matrix dispersion droplets and thus minimising their size.

Table 1. Effect of stirring speed on the general characteristics of agarose based microcapsules prepared by w/o emulsion using as continuous phase isopropyl myristate

batch	agarose (%, w/v)	stirring speed (r.pm)	mean size (µm)	SD	notes
AgEm-1	2%	500	645	98	Spherical microcapsules with moderate size dispersion some irregularities
AgEm-2	2%	600	431	76	Spherical microcapsules with moderate size dispersion
AgEm-3	2%	700	248	52	Spherical microcapsules with moderate size dispersion

Table 2. Effect of stirring speed on the general characteristics of agarose based microcapsules prepared by w/o emulsion using as continuous phase labrafac CC

batch	agarose (%, w/v)	stirring speed (r.pm)	mean size (µm)	SD	notes
AgEmL-1	2%	550	338	60	Spherical microcapsules with moderate size dispersion
AgEmL-2	2%	600	239	51	Spherical microcapsules with narrow size distribution
AgEmL-3	2%	650	211	52	Spherical microcapsules with moderate size dispersion
AgEmL-4	2%	700	209	47	Spherical microcapsules with moderate size dispersion

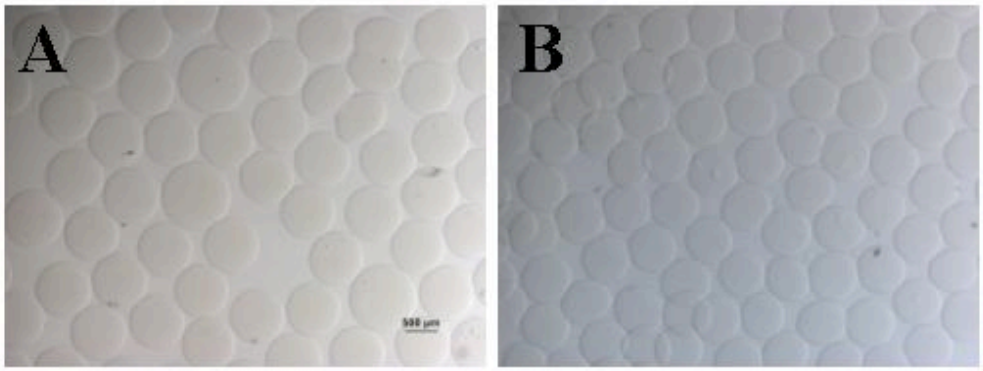

Fig. 3. Effect of surfactant concentration rate on the morphological characteristics of the agarose microparticles. Stereomicrographs of agarose microcapsules manufactured by w/o emulsion using as continuous phase isopropyl myristate. Samples were prepared with a surfactant concentration of 1.0 (A) and 1.5 %(w/v) (B).

Decreasing the polymer concentration from 2% to 0.8%, resulted in the formation of smaller microparticles. In fact, decreased viscosity of the matrix dispersion (depending on polymer concentration) yields smaller microspheres since lower shear forces are necessary for droplet disruption. Spherical agarose microparticles were formed with agarose 1-2% (w/v) but at lower concentration (0.8% w/v) some elliptical microparticles were produced, probably because the warm agarose at this low concentration deforms prior to gelation.

The effect of manufacturing parameters on the size of agarose microparticles prepared by emulsion method was investigated. Smaller microparticles with a narrower size distribution were obtained when the stirring rate and surfactant concentration were increased while decreasing polymer concentration.

References

Craige J. S. et al. (1990). *Biology of red algae*. Cambridge University Press 221-57
Iwata H. et al. (1986). *In vitro and in vivo evaluation of microcapsulated islets as hybrid artificial pancreas*. Jpn J Artif Organs 15:1493–6.
Tun T. et al. (1996). *A newly developed three-layer agarose microcapsule for a promising biohybrid artificial pancreas: rat to mouse xenotransplantation*. Cell Transplant 5 S59–63.
M. Miyamoto Y.J. et al. (2000). *Effect of neovascularization-inducing bioartificial pancreas on survival of syngeneic islet grafts*. Transplant Proc 32 2494–5.
Nilsson K. et al. (1987). Mosbach. *Entrapment of microbial and plant cells in beaded polymers*. Methods in Enzymology, Immobilized Enzymes and Cells, Part B.135 222–230.
Mu Y. et al. (2005). *Manufacture by water/oil emulsification of porous agarose beads: effect of processing conditions on mean particle size, size distribution and mechanical properties*. Chem. Engineering and Processing 44 1157-1166
Freitas S. et al. (2005). *Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microsphere preparation process technology*. J Control Release 102 313–332.