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Thermosensitive Microparticles Based on Unsaturated Esters of some Poly- and Oligosaccharides: Preparation, Characterization, Drug Inclusion and Release

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Summary: The paper concerns the preparation and characterization of hydrogel microparticles based on exopolysaccharide (Gellan, Xanthan) unsaturated derivatives and respectively on cyclodextrin as well as their application for some hydrosoluble and liposoluble drugs inclusion. In the first step the polysaccharide and cyclodextrin unsaturated esters (maleate, acrylate) were synthesized and their hydrogel forming capacity was tested using a grafting-crosslinking free-radical reaction with N-isopropyl acrylamide (NIPAm), at room temperature. For a better control of the crosslinking degree N,N' methylene-bis-acrylamide (BIS), replaced by cyclodextrin tri-acrylate (A-CD) in a few experiments, was used. The microparticles were obtained by using the method in w/o emulsion, in which the dispersed aqueous phase is the reaction mixture and the oil phase is hexane. The particles containing polysaccharide esters showed an average diameter around 100 μm when crosslinking was achieved with BIS. They were smaller than those crosslinked with A-CD, which are in the range of 200-300 μm; the particles based on Xanthan maleate were smaller than Gellan maleate based ones. Even much smaller particles (2-2.5 μm in diameter) were obtained by starting from A-CD grafted-crosslinked systems. The synthesized microparticles are able to include chloramphenicol, as well as progesterone; the drug is slowly released according to diffusion controlled kinetics. The application of these microparticles in emergency ophthalmic treatments is possible as a result of their thermal sensitivity; they can collapse and release the drug instantly when placed in contact with the human eye, at 37 °C.

Keywords: cyclodextrin; drug release; microparticles; polysaccharide; thermosensitive hydrogel

Introduction

Peptides, proteins, oligonucleotides, genes and some drugs are relatively unstable compounds in physiological medium and need to be protected when administered into organism. Their efficiency is, often, limited by their incapacity to cross some biological barriers to reach the target. As a consequence, the elaboration of adequate vehicles, capable to include, to carry, to protect and finally to deliver in a controlled manner the biologically active compounds is necessary.

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Several strategies were explored to realize this kind of vehicles, a great attention being devoted to micro- and nanoparticles. The use of biodegradable hydrophobic polymers, [poly(lactic acid), poly(glycolic acid), poly(ε-caprolactone), poly(hydroxy alkanoates)],[1–5] and later on amphiphilic polymers [poly(lactide-co-glycolide), poly(lactide)-poly(ethylene glycol)][6–8] appeared as a great promise. Obtaining of such systems requires, in most of the cases, aggressive conditions (organic solvents, high temperature, sonication etc), which may compromise the stability of the included molecules.

For instance it would be advantageous to combine hydrophilic and hydrophobic polymers to design hydrogels for drug delivery. The hydrophobic polymer may act as drug carrier, whereas the hydrophilic one ensures biological interactions. In view of these aspects, the polysaccharides, as reported in the recent review article of Prabaharan and Mano[9] present an attractive and important alternative due to their biocompatibility, biodegradability, hydrophilicity, biomimetic physicochemical properties, and capacity to be chemically modified through graft functionalization[10–12] and hydrophobisation.[13–15]

Among the polysaccharides used for this goal, an important place is occupied by Xanthan and Gellan, because they are forming both physical and chemical gels, the latter ones by several methods.[16–18]

A tremendous amount of research is devoted to the preparation of novel pH and thermosensitive hydrogels for biomaterials and physiological applications. Among the various families of thermosensitive polymers, alkyl acrylamide-based polymers, in particular poly(N-isopropylacrylamide) (PNIPAm), are the most investigated for these applications. The choice of NIPAm was justified in order to obtain hydrogels sensitive to external stimuli such as temperature. The biocompatibility of PNIPAm systems has increased the success of this family of polymers, such as for dextran-PNIPAm hybrid hydrogels described by Kim et al.,[19] Zhang et al.,[20] and for Xanthan-PNIPAm hydrogels of low Xanthan contents, quite recently mentioned by Long et al.[21] In the same time, the ability of NIPAm based networks to change their conformation at temperatures larger than 32ºC (LCST) is well known.[22,23]

This work presents the preparation of new thermosensitive hydrogel based particles designed for the inclusion of hydrophilic or/and hydrophobic drugs.

Access to these particles is based on a new concept, checked previously with bulk hydrogels:[24] first the polysaccharide functionalization by introduction of double bonds as substituents of the polymer backbone. Then it was followed by crosslinking of obtained polysaccharidic macromer by a free-radical grafting/crosslinking reaction of an acrylic monomer (NIPAm).

Our intention was therefore to associate NIPAm monomer with a polysaccharide to obtain a compound having hydrogel properties as well as thermosensitive characteristics. Moreover its transition temperature has to be as close as possible to that of human body.

Another goal was the preparation of an hydrogel able to include either water soluble or liposoluble drugs. It was well known that cyclodextrins, thanks to their particular structure, have the ability to include liposoluble compounds within the hydrophobic cavity.[25–29] As a consequence it was proposed to find a way to chemically bind such an oligosaccharide within the networks based on modified polysaccharide and NIPAm. The chosen reaction path was to functionalize the oligosaccharide introducing unsaturated substituents, leading to cyclodextrin acrylates.

Further, preparation of crosslinked structures was carried out by a free-radical grafting/crosslinking reaction of the polysaccharide unsaturated ester with NIPAm, at room temperature. As a general rule, NIPAm was grafted to the polysaccharidic chain forming macroradicals, which afterwards, by specific termination reactions, mainly by recombination, lead to grafted or crosslinked structures. Referring to these

termination reactions, the NIPAm chains will constitute bridges between two polysaccharidic chains.

The control of the crosslinking degree, in order to obtain networks with various swelling capacities within physiologic liquids, respectively various capacities of drug inclusion, was achieved by crosslinking either with N,N'-methylene bisacrylamide or with acrylate cyclodextrin.

Experimental Part

Materials

Xanthan gum (from *Xanthomonas Campestris*) with a M_w of 2.6×10^6 g/mol[17] was obtained from BioChemika (degree of substitution per side chain of 0.73 and 0.75 for acetate and pyruvate groups, respectively, as determined by proton NMR). Precursor Gellan gum with a M_w of 2.35×10^6 g/mol[30] was obtained from CPKelko. β-cyclodextrin was supplied by Wacker-Chemie GmbH, Burghausen, Germany. Maleate Xanthan (MA-X) (with a degree of substitution, DS of 10.8%), maleate Gellan (MA-G) (DS of 15.4%) and β-cyclodextrin acrylate (A-CD) (with a DS ≅ 3) were synthesized and characterized according to our previous published procedures.[13,14,31] N-isopropylacrylamide (NIPAm), N,N'-methylene bisacrylamide (BIS), N,N,N',N'-tetramethylethylenenediamine (TEMED), were purchased from Sigma-Aldrich and used as received. Ammonium persulphate (APS) was obtained from Merck. Span 80 (sorbitan monooleate), hexane anhydrous, Chloramphenicol hemisuccinate and Progesterone were purchased from Sigma-Aldrich.

Preparation of Particles

Microparticles were synthesized using a reverse emulsion (w/o), from the dispersion of the aqueous phase (polysaccharide or oligosaccharide ester, NIPAm, crosslinking agent, initiator and activator) within the organic phase (hexane). Stabilization of emulsion was performed using Span 80 as surfactant.

The chemical process to elaborate these hydrogel microparticles, as an interpenetrated network, was based on the free-radical grafting/crosslinking reaction of polysaccharide maleate with NIPAm (APS; TEMED as initiator) in presence of a crosslinking agent (either BIS, or A-CD) within the aqueous droplets. The crosslinking of A-CD was processed in a similar manner, but without the introduction of an additional crosslinking agent.

Four kinds of microparticles were obtained; their code and the compositions of reaction medium were shown in Table 1.

The ester aqueous solution (c = 2.5%) was initially prepared. A-CD (or BIS) was added under magnetic stirring for 24 hours, then the initiator (APS, 3.5% g/g with respect to co-monomers mixture). Stirring was pursued during one hour. The solution was transferred in a container fitted with a septum equipped screwtop and degassed with nitrogen during 20 minutes to eliminate oxygen from the reaction medium. The activator, TEMED (1.1 mol/1mol of APS) was then added using a syringe and the solution finally transferred in a reactor and dispersed under strong stirring (800 rpm) within hexane in the presence of surfactant (Span 80, 2%, v/v) chosen according to HLB of the mixture.[33,34] The aqueous solution/hexane volume ratio was of 1/9 (emulsion total volume of 200 ml). The reaction was carried out at room

Table 1.
Composition of the reaction mixture for obtaining microparticles (concentration of the aqueous solution of poly(oligo)saccharide ester: 2.5%).

Sample code	MA-PZ/NIPAm[a] ratio, (%, g/g)		BIS[b], (%, g/g)	A-CD[b], (%, g/g)
	MA-PZ	NIPAm		
NC	–	100	–	3
XNC	25	75	–	3
GNB	25	75	3	–
GNC	25	75	–	3

[a]MA-PZ: polysaccharide maleate (X - Xanthan ; G - Gellan); N - NIPAm; B - BIS; C - A-CD); [b]with respect to polysaccharide maleate/NIPAm mixture; Initiator: APS (3.5% g/g with respect to polysaccharide maleate/NIPAm mixture) ; Activator: TEMED (1.1 mol TEMED/1mol APS).

temperature (20 °C; T< LCST) during 24 hours. At the end of the reaction, the microparticles were separated by centrifugation and submitted to several washings succesively with water, aceton and with tetrahydrofurane (THF). Drying was performed first at room temperature (72h) and finally under vacuum (48 h).

Characterization Techniques

Dimension of Particles

The mean diameter and the polydispersity of the particle dimensions were determined using a Shimadzu-SALD 7001 laser diffraction analyzer. It is able to measure the size of particles in suspension within the 15 nm - 500 μm range. The analysis was carried out on microparticles dispersed into tetrahydrofuran to avoid their swelling, leading to sizes close to dry microparticles. Measurements were performed under continuous stirring, at ambient temperature and series of five analysis were performed on each sample. The incident laser beam wavelength was 405 nm.

Scanning Microsocopy

Scanning electron microscopy (SEM) was used to study the morphology of the microparticles. Samples, beforehand gold-coated, were analyzed using SEM Philips 525M.

Swelling Studies

Maximal swelling degree of the hydrogels was determined by thermogravimetry using TA 2950 apparatus. Temperature was increased according to a continuous ramp (10 °C/min) from 30 to 120 °C, followed by an isotherm at 120 °C during 60 minutes, in air. Dry particles were first immersed within the swelling medium (water, ethanol) during 24 hours. Then, the excess of solvent was removed by soft swabing with a filter paper. Swollen particles were placed inside the aluminum crucible before beginning the temperature program. The swelling ratio was determined from the relation :

$$Q_m = \frac{m\text{-}m_0}{m_0}100 = \frac{m_1}{m_0}100(\%)$$

where Q_m is the swelling degree (%, in mass), m_0 the weight of dry hydrogel (g), m the sample weight after swelling (g), and m_1 the weight of swelling agent retained by sample (g).

Determination of Drug Loading

The capacity of inclusion/release of drugs in/from the hydrogel based microparticles was determined by UV-Vis spectroscopy (UV-2101PC spectrophotometer from Shimadzu), at 276 nm for chloramphenicol and 241 nm for progesterone. Calibration curves were plotted from drug solutions at known concentrations (within 10^{-6} – 10^{-5} g.ml^{-1} range), and absorbance coefficient was determined.

Inclusion of drugs was carried out by diffusion in an exact weight of microparticles (around 0.2 g) beforehand swollen up to equilibrium in the solvent (water, alcohol) and then dispersed in 50 mL of the drug solution (concentration 1 g.l^{-1}). After 72 h, 0.5 mL of supernatant were removed ; after dilution up to 10 mL the absorbance was measured. From the calibration curves the drug concentration of the supernatant was measured and hence the quantity of drug included in the support.

Determination of in vitro Drug Release

The same process was used to study the drug release kinetics. The microparticles loaded with drug were centrifuged and, after removal of the supernatant, they were suspended in 50 mL of distilled water. Periodically, constant volumes of supernatant were removed (0.5 mL), diluted up to 10 mL and the quantity of released drug was calculated from the spectrophotometric calibration curves. The release kinetics was followed in static regime.

Results and Discussion

The structure of the final products resulting from the ternary ''macromer-NIPAm-BIS (A-CD)'' system is extremely complex, implying on one hand the polysaccharide network crosslinked with NIPAm bridges and BIS, and on the other hand the NIPAm

network directly crosslinked with BIS, with both networks being formed simultaneously.

Polysaccharide maleates and NIPAm-based hydrogels, implying BIS as the crosslinking agent, which form the microparticles may be considered as full-interpenetrated networks (IPN).

For an hydrogel based on Gellan maleate (GNB, Table 1), the structure may be represented as follows in Scheme 1.

When BIS was replaced by cyclodextrin acrylate as crosslinking agent (XNC, GNC, Table 1) the corresponding structure is represented in Scheme 2.

If the polysaccharide maleate does not participate to the formation of the network, the structure was as follows (Scheme 3), considering it was only formed with NIPAm and A-CD (NC, Table 1).

Electron microscopy photographs of microparticles based on polysaccharide maleates (Figures 1, 3 and 4) show that they are not spherical and of irregular shape. Their size depends upon the composition of the comonomers mixture (polysaccharide macromer and NIPAm).

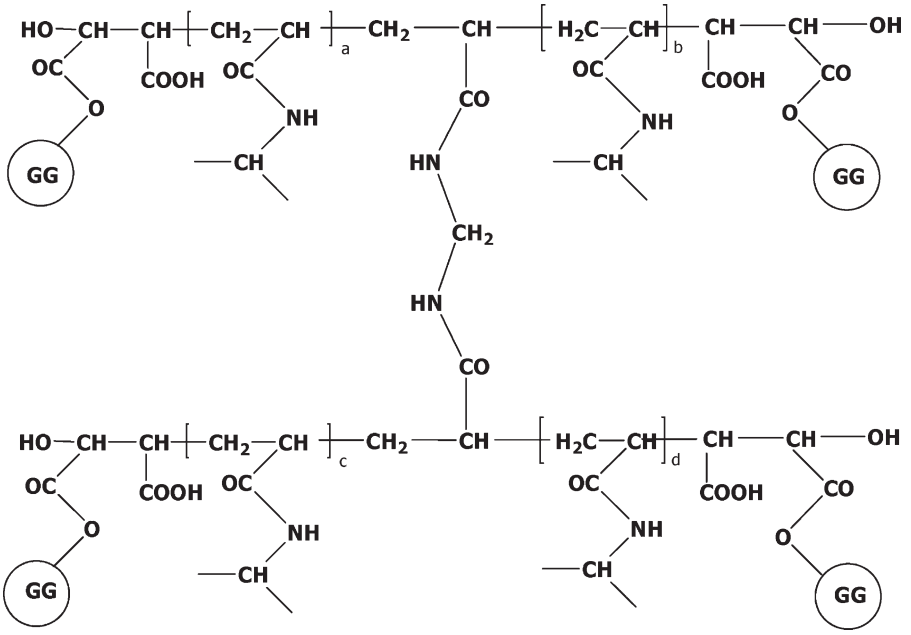
The average diameters of microparticles based on the Gellan ester are around of 100 μm as observed on Figure 1 and their structure was compact.

The polydispersity in size was quite broad. This was confirmed from the laser diffraction analysis of these microparticles. The differential and the integral curves are given in Figure 2. The size domain was between 30 and 300 μm, and the average diameter was equal to 93 μm.

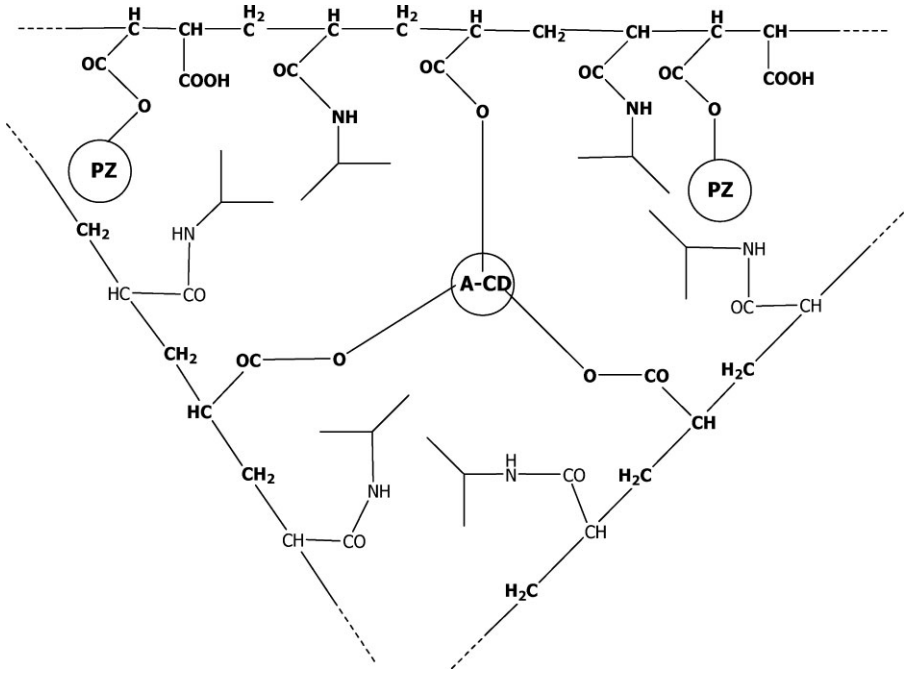
When BIS was replaced by A-CD as crosslinking agent, obtained microparticles have a larger mean diameter and a more porous structure as observed on Figure 3.

The mean diameter estimated from the scanning electron microscopy photographs was around 200 μm.

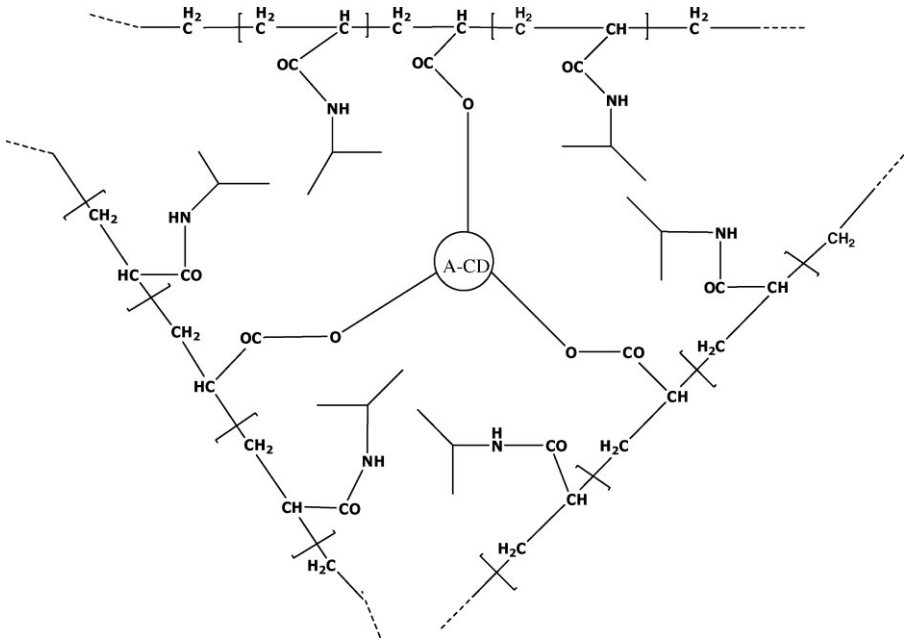
For the same starting comonomers mixture the microparticles obtained with Xanthan maleate (sample XNC) are smaller than those prepared under the same conditions with Gellan maleate (sample GNC). These XNC microparticles have a strong tendency to aglomerate on drying. As these aglomerates are difficult to be



Scheme 1.
Structure of a network based on Gellan maleate and NIPAm crosslinked with BIS.



Scheme 2.
Structure of a network based on polysaccharide maleate and NIPAm crosslinked with A-CD.



Scheme 3.
Structure of a network based on NIPAm crosslinked with A-CD.

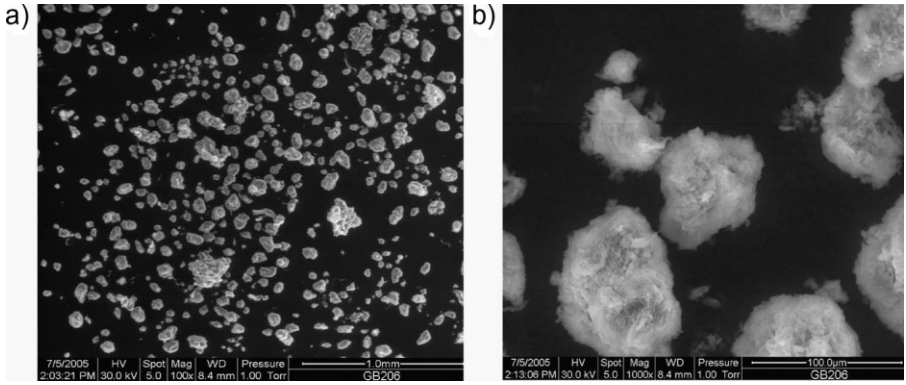


Figure 1.
Scanning electronic microscopy micrographs for microparticles based on GNB type hydrogels; a – x100; b – x1000.

redispersed in THF, their size distribution could not be determined accurately.

The last type of microparticles synthesized were based on NIPAm crosslinked with cyclodextrine acrylate, A-CD. The difference in the mean diameter of these microparticles with respect to those based on polysaccharides is of two orders of magnitude, perhaps due to the lowest viscosity of the aqueous solution submitted to the crosslinkling reaction, leading to its better dispersion in the organic phase. Figure 5 shows an average value of arround 2.3 μm with a relative narrow size distribution, between 1.5 – 4 μm.

The smaller sizes of these microparticles were confirmed by scanning electronic microscopy (Figure 6). From the morphological point of view these particles seem to be composed of other aggregated spherical nanoparticles. Voids between these nanoparticles induce a higher porosity than the one observed for polysaccharide maleate based microparticles.

The capacity of microparticles to include drugs is strongly related to swelling

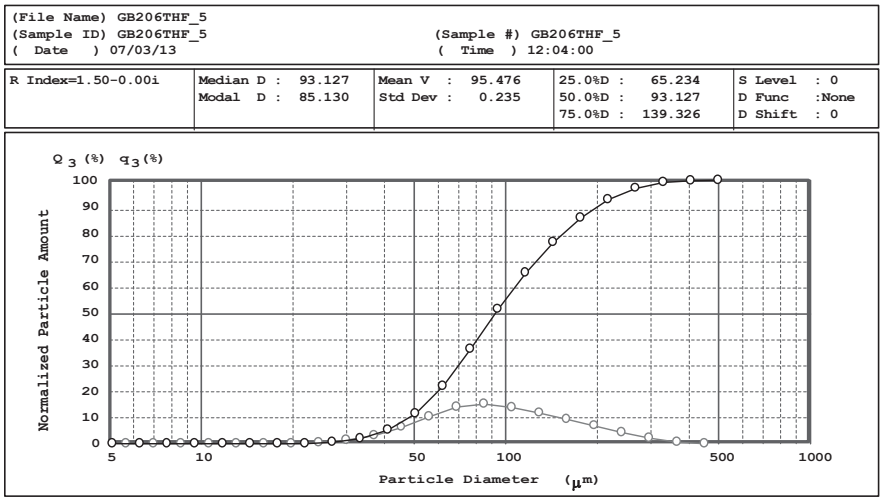


Figure 2.
Size differential, respectively integral distribution curves of microparticles based on GNB type hydrogels.

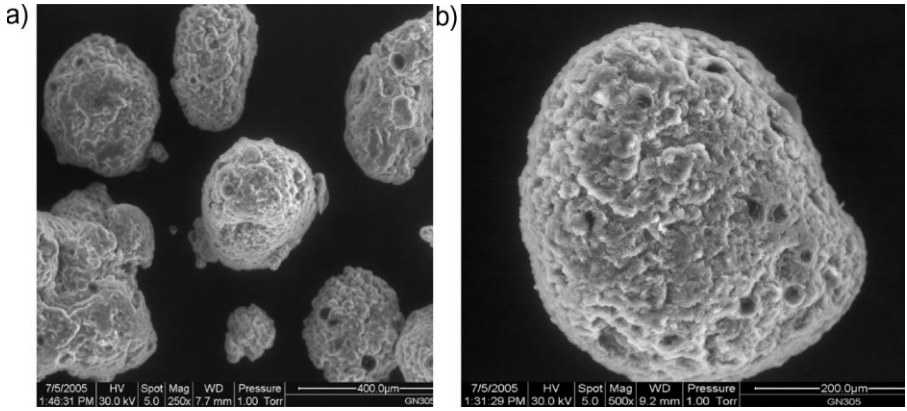


Figure 3.
Scanning electronic microscopy micrographs for microparticles based on GNC type hydrogels; a – x 250; b – x 500.

properties of the hydrogel from which they are processed (maximal swelling ratio, swelling kinetics constant). The maximal swelling degree (Q_{max}) of microparticles is given in Table 2 in comparison to the corresponding bulk materials prepared under the same composition.[24,30]

From this table it appears clearly that the swelling tendency is quite similar for microparticles and the corresponding bulk material. The slightly lower swelling almost within the experimental errors limits may be attributed to the interfacial effects well known for microparticles swelling.[32]

For both systems, microparticles and bulk materials it can be noticed by comparing sample NC with all the others, that the swelling degree is systematically higher for all samples containing polysaccharide. This is a direct evidence that on replacing 25% of PNIPAm by the corresponding weight amount of olysaccharide, which is much more hydrophilic than PNIPAm, the swelling of the hydrogel increases dramatically. This enhanced swelling, due to the hydrophilicity of the polysaccharide in the IPN structure, appears in spite the fact that the maleate

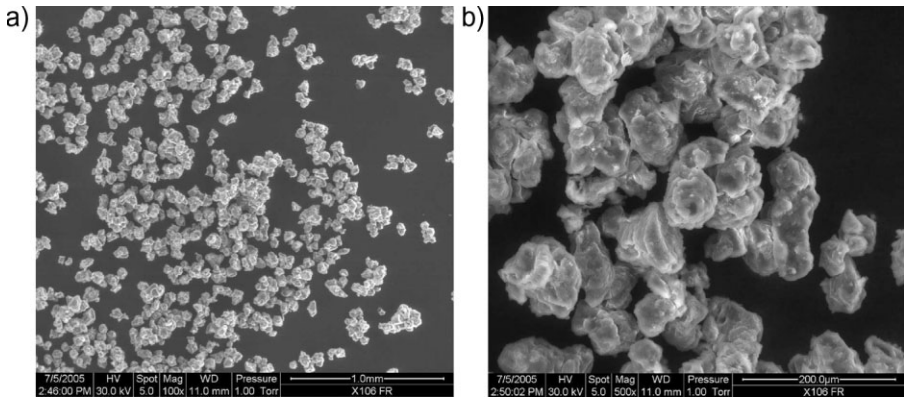


Figure 4.
Scanning electronic microscopy micrographs for microparticles based on XNC type hydrogels; a – x 100; b – x 500.

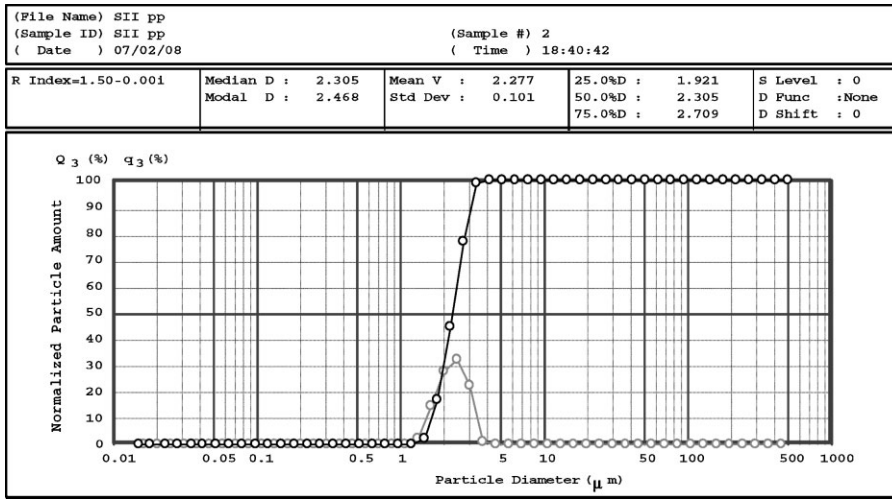


Figure 5.
Size differential, respectively integral distribution curves of microparticles based on NC hydrogel.

polysaccharide acts as a crosslinker in addition to NIS and CD respectively. As only minor differences in the swelling behaviour is observed for samples GNC and GNB, it might be concluded that acrylate CD and BIS have similar crosslinking efficiency.

On a first sight, it seems surprising as well for microparticles as for bulk materials with the same composition that Xanthan based hydrogels (XNC)having 1.18 double bonds per repeating unit present a lower Qmax value than those based on Gellan (GNC) with 1.54 double bonds per repeating unit, with the same composition. This apparent discrepancy in swelling behaviour of samples XNC and GNC having similar molar weights may be explained by the fact that the swelling degree is directly related to the average distance between 2 crosslinking points of the main chain. Hence Xanthan has 2 glucosidic units per repeating unit inthe main chain, whereas Gellan has 4. Therefore, the main chain, where the maleate unjts are fixed, has on the average $1.18/2 = 0.6$ double bonds per glucosidic unit in the case of Xanthan and only $1.54/4 = 0.4$ for Gellan. The crosslink density is consequently higher (and swelling lower) for XNC than for GNC.

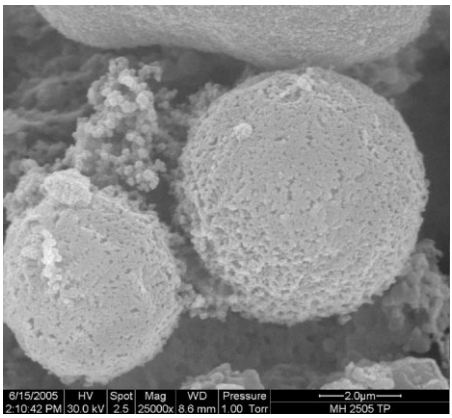


Figure 6.
Scanning electronic microscopy micrograph for microparticles based on NC type hydrogels (x 25.000).

Table 2.
Maximal swelling degree in water (Q_{max}) of microparticles and bulk materials.

Codes	Q_{max} hydrogels bulk material (g/g)	Q_{max} microparticles (g/g)
GNB	17.7	16.8
GNC	19.0	17.5
XNC	11.6	10.3
NC	7.0	6.25

Table 3.
Quantity of CLF included and released after 72 hours by XNC and GNC based microparticles.

	XNC	GNC
CLF included, (mg/g dry microparticles)	22.1	27.1
CLF released (mg/g dry microparticles)	17.1	18.7

The higher porosity and the less rigide structure of the GNC based microparticles as compared to XNC based ones may contribute somehow to the swelling degree increase.

The capacity of the microparticles to include water soluble drugs was demonstrated using chloramphenicol (usually considered as a model water soluble drug, CLF) and it was in agreement with the swelling behaviour : Gellan based microparticles include, respectively release, higher quantities of drug, compared to Xanthan based ones (Table 3).

Release kinetics curves show the typical profile of diffusion controlled release systems (Figure 7).

The amount of released drug was maximal after around 24 hours and then remains constant. Considering the release process, a ''burst effect'' was observed during the first three hours. It might be expected that the inclusion of other water soluble drugs, such as adrenaline, leads to similar results, opening the prospect to use such microparticular systems for ophthalmic applications and in particular in the ophtalmic emergency treatment for its vasoconstrictor effects.[35]

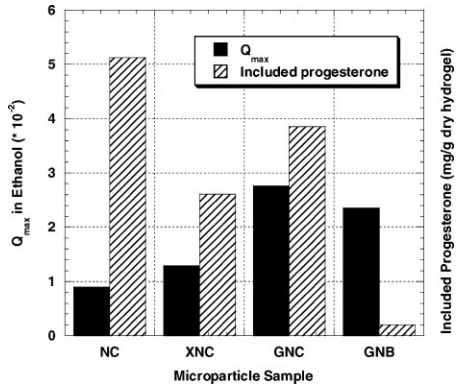


Figure 8.
Maximum swelling degree in Ethanol and maximum progesterone amount included in different type of microparticles.

The incorporation of cyclodextrin acrylate as a crosslinking agent allows the use of the microparticles to include liposoluble drugs. As an example, progesterone, which is slightly soluble in water but well soluble in ethanol, was tested for inclusion in hydrogels immersed in its alcoholic solution. Preliminary results are given in Figure 8 demonstrating the interest of such hydrogels for inclusion of liposoluble drugs, such as progesterone, which is of particular interest for ophthalmic applications as for instance in glaucoma treatment.[36]

From these tests it turns out that the presence of CD, by its hydrophobic cavity, has a very beneficial effect on the progesterone inclusion.

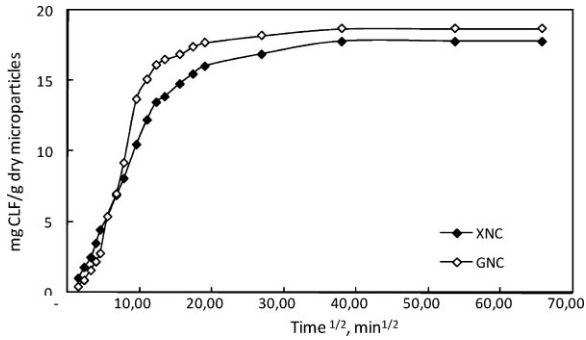


Figure 7.
CLF Release kinetics curves of GNC and XNC based microparticles.

The thermosensitive character of the hydrogels as bulk materials was previously demonstrated.[24,30] These materials were able to collapse at temperature higher than the LCST of poly(NIPAm), and similar behaviour is therefore expected for the microparticles of same composition. Such a thermostimulable effect on drug loaded microparticles would be of major interest for ophtalmic applications.

Further details of the medical tests performed with progesterone loaded hydrogels will be given later.

Conclusion

Microparticles with hydrogel character based on Xanthan maleate/ N-isopropylacrylamide were elaborated by a grafting-crosslinking reaction using either N,N'-methylenebisacrylamide (BIS) or cyclodextrin acrylate (A-CD) as crosslinking agent. The microparticle diameter depends on the chemical nature of components, and is within the 2.5-400 μm range

The swelling ability and the inclusion/ release of water soluble drugs depend on the hydrophily and stiffness of presursor polysaccharide. The microparticles containing β-cyclodextrin in their composition are able to load liposoluble drugs.

Finally, the presence of PNIPAm within the hydrogel microparticles imparts them a thermosensitive character which, in combination with the capacity to load lipophile drugs, opens interesting prospects in ophthalmic applications.

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