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An improved method for preparing glutaraldehyde cross-linked chitosan–poly(vinyl alcohol) microparticles

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Abstract The features of microparticles, as size, surface structure, and morphology, depend, mainly, on the methodology used for their preparation. Emulsion polymerization techniques are undoubtedly among the most widespread. However, the use of toxic, volatile organic solvents represents a major disadvantage, namely, because of environmental issues. In this study, we prepared glutaraldehyde cross-linked chitosan–poly(vinyl alcohol) microparticles by an improved water-in-oil emulsion technique using corn oil as organic phase. The application of this polymeric blend as microparticle is scarcely investigated. As resulting of the procedure here presented, spherical and smooth surface microparticles were obtained, with mean diameter of 16 μm. The cross-linking reaction between the aldehyde and the amino or the hydroxyl groups formed either an imine (Schiff's base) or an acetal bond, respectively, as analyzed by infrared spectroscopy. The microparticles here described did not present cytotoxic potential. Accordingly, this study can find promising and successful application in biotechnology.

Keywords Microparticle · Chitosan · Poly(vinyl alcohol) · Glutaraldehyde · Water-in-oil emulsion · Cytotoxicity

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Introduction

The development and/or improvement of polymeric matrices represents an interesting and promising challenge in Material Sciences. Strategies as chemical modification or combination of polymers can be straightforwardly followed towards new materials with potential applications in several fields. In the case of biomaterials, improvements in theirs characteristics have been achieved by the combination of synthetic polymers and biological polymers. Whereas synthetic polymers are characterized by their good physicochemical and mechanical properties but not sufficient biocompatibility, biological polymers have good biocompatibility but their mechanical properties are often limited [1]. An example of a successful combination has been blending chitosan and poly(vinyl alcohol) (PVA) [2].

Chitosan is a natural cationic polysaccharide, which is a copolymer formed by units of 2-deoxy-*N*-acetyl-D-glucosamine and 2-deoxy-D-glucosamine linked by β-1,4 glycosidic bonds. It occurs rarely in nature being generally obtained by alkaline and by enzymatic deacetylation of chitin, the second most abundant natural polymer found in nature, obtained from the exoskeleton of shrimps, fungi, insects, annelids, and mollusks. Chitosan has been preferred over chitin because it is readily soluble in dilute acids and, therefore, easily processed. Moreover, chitosan is biocompatible, biodegradable, nontoxic, mucoadhesive, hemostatic, hypocholesterolemic, hypolipidemic, antimicrobian, immunoadjuvant, antiviral, and antitumoral [3], which make it very attractive for applications in Medicine and Pharmacy. This biopolymer has been also tested in other scientific and industrial fields as soil remediation [4], wastewater treatment [5], food packaging [6] and cosmetics [7], among others.

PVA is produced by the polymerization of vinyl acetate to poly(vinyl acetate) (PVAc), followed by hydrolysis of PVAc to PVA. The hydrolysis reaction is not complete resulting in polymers with a certain degree of hydrolysis that depends on the extent of reaction [8]. As it is nontoxic, water-soluble, biocompatible, and biodegradable, this synthetic polymer is widely used namely for biochemical and biomedical applications [9].

There are several chitosan cross-linking agents already described such as genipin [10], glyoxal [11], epichlorohydrin [12], and ascorbic acid [13]. In this study, glutaraldehyde was chosen as cross-linking agent. Glutaraldehyde has been used more frequently as a cross-linking agent than any other reagent, as it is less expensive, readily available, and highly soluble in aqueous solution [14]. The high reactivity of the aldehyde groups, which readily form imine bonds (Schiff's base) with amino groups and acetal bonds with hydroxyl groups [15], provides the efficiency of glutaraldehyde on the cross-linking of chitosan and PVA.

The polymeric matrix resultant from the cross-linking of chitosan and PVA by glutaraldehyde has shown promising attributes for practical applications, because of its high swelling and shrinking ratio, high pH sensitivity and biodegradability [16]. Membranes [17], hydrogels [16], and films [2] based on this polymeric matrix were reported. From our knowledge, our research group prepared, for the first time, microparticles based on a similar polymeric matrix [18].

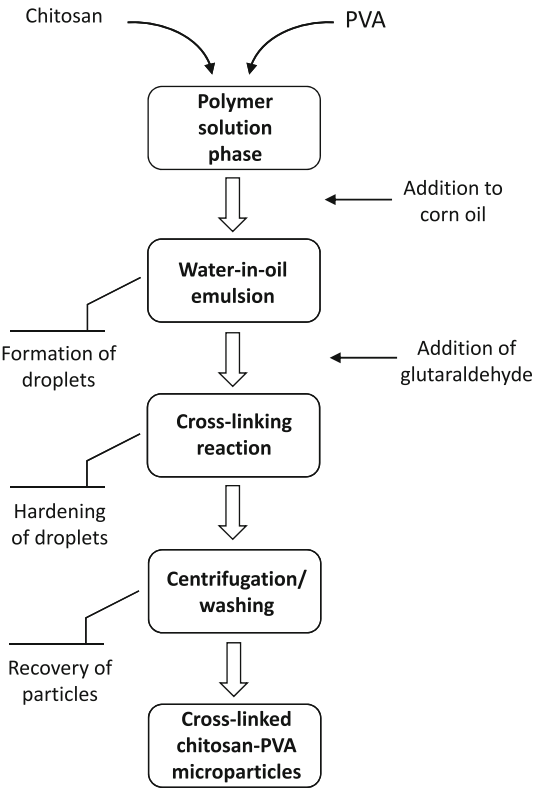
Microparticles constitute a technology used by several industries as agricultural, food, graphics, cosmetics, and pharmaceutical. For the preparation of these systems, there is a large number of starting materials and techniques [19]. In this study, microparticles were prepared by solvent extraction/evaporation, specifically water-in-oil emulsion. Basically, this technique consists of four major steps: (i) dissolution of the polymer in an appropriate solvent; (ii) emulsification of this polymeric phase in a second continuous phase immiscible with the first one; (iii) extraction of the solvent from the dispersed (polymeric) phase, transforming the droplets into solid microparticles; (iv) recovery of the microparticles. In water-in-oil emulsion, neither elevated temperatures nor phase separation-inducing agents are required [20], which make it valuable for industrial applications and suitable for the encapsulation of thermolabile compounds. However, the use of toxic, volatile organic solvents as the continuous phase such as cyclohexane [21], toluene, and chloroform [22] constitutes a major drawback regarding the safe production and usage of the microparticles. To overcome this issue, we developed a nonvolatile organic solvent water-in-oil emulsion method using vegetable oil (corn oil) as organic phase.

In this study, we improved a previous procedure [18] aiming the design of a water-in-oil emulsion technique protocol volatile organic solvent-free to prepare glutaraldehyde cross-linked chitosan–PVA microparticles (CCPMs). Moreover, we also improved the procedure to obtain smooth surface microparticles. Particle size analysis, morphological and topographical studies were made, as well as chemical studies by attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy. As we were mainly focused on an application in the field of biotechnology and biomedicine, cell toxicity of CCPMs was also evaluated.

Experimental section

Materials

Chitosan (medium molecular weight, degree of deacetylation 75–85 %), glutaraldehyde (25 % (v/v)) aqueous solution and PVA (M_w 9,000–10,000, 80 % hydrolyzed) were obtained from Aldrich (St. Louis, MO). Acetic acid (96 %, p.a.) and methanol (p.a.) were purchased from Merck (Darmstadt, Germany) and Panreac (Barcelona, Spain), respectively. Corn oil and petroleum ether were received from Sovena (Algés, Portugal) and JM Vaz Pereira (Sintra, Portugal), respectively. Complete RPMI 1640 was used as culture medium and was prepared with RPMI 1640 (Invitrogen, Carlsbad, CA), penicillin–streptomycin solution (1 %), L-glutamine (1 %), and 10 % (v/v) fetal bovine serum were purchased from Aldrich (St. Louis, MO). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was supplied by Aldrich (St. Louis, MO). Isopropanol and chloridric acid were bought from Merck (Darmstadt, Germany). All chemicals were used without further purification. The distilled water used was prepared in the laboratory. Sterile 96-well flat-bottomed culture plates were supplied by Corning Inc, Costar® (Lowell, MA).



Scheme 1 Representative steps followed for the preparation of CCPMs by water-in-oil emulsion method

Preparation of CCPMs by water-in-oil emulsion

The main steps of the preparation of CCPMs by water-in-oil emulsion are depicted in Scheme 1. The dispersed/polymeric phase was composed by 2 % (w/v) chitosan solution in 2 % acetic acid:methanol (2:1, v/v) and 2 % (w/v) PVA in water:methanol (1:2, v/v). This aqueous polymeric solution was added, dropwise, to corn oil as organic continuous phase (1:50, v/v). A stable water-in-oil emulsion was obtained after 2 h at 38 °C, under continuous mechanical stirring (RZR-1, Heidolph, Germany) at 1,370 rpm. The cross-linking agent, 25 % (v/v) glutaraldehyde as solution saturated in toluene [23–25] was dropped in the emulsion. The cross-linking reaction was carried out for during 2 h at 38 °C. The CCPMs obtained were recovered from the continuous phase by centrifugation at 3,500 rpm for 10 min. Then, the CCPMs were washed with petroleum ether twice and copiously with distilled water. Finally, CCPMs were dried in the oven at 37 °C and kept in sealed containers prior to their use.

Morphology

All CCPMs were preliminarily observed for size and shape by optical microscopy using a BH2 (Olympus, Japan) microscope. Shape and surface characteristics of

CCPMs were investigated by scanning electron microscopy (SEM). The CCPMs were dispersed in water, dropped in a cover glass, and air-dried at room temperature. The cover glass was fixed onto a metal stub with a double-sided adhesive tape. The sample was then sputter coated, under vacuum and in argon atmosphere, with copper and examined in a JSM-5310 (Jeol, Japan) scanning microscope operating at 25 kV, at room temperature.

Particle size study

The particle size and particle size distribution were analyzed by laser light scattering using a LS 130 (Beckman Coulter, USA) particle analyzer. Small amounts of CCPMs were suspended in water. To destroy possible aggregates, CCPMs were sonicated for 1–2 min before analysis. Particle size and particle size distribution values were determined according to the Theory of Fraunhofer, using the Coulter software.

FTIR analysis

Chemical composition of CCPMs polymeric matrix was analyzed by infrared spectroscopy using a Magna IR System 750 (Nicolet, USA) IR spectrometer with an ATR accessory (golden gate Mk II with diamond top-plate and ZnSe lenses, Specac, UK). Chitosan, PVA, and CCPMs FTIR spectra were recorded at room temperature, in the frequency range 4,000–400 cm^{-1}, at 4 cm^{-1} resolution and scanned 32 times.

Cell toxicity

The cytotoxicity of CCPMs was investigated by MTT assay [26] using peritoneal macrophages harvested from Wistar mice. Wistar mice (aprox. 300 g) were obtained from the Animal Facility of Faculty of Medicine, University of Coimbra, Portugal. All animals were maintained in accordance with to the national and international regulations on animal welfare. Macrophages were cultured in complete RPMI 1640 medium, at 37 °C in a humidified atmosphere containing 5 % CO_2, for 4 h, until form a confluent monolayer [27]. Afterward, culture medium was replaced by CCPMs suspensions in complete RPMI medium at 0.2, 2.0, and 10 % (v/v) (final concentration). As negative control, the cells were incubated in complete culture medium without CCPMs. The cells were incubated in the same conditions. After 20-h incubation, the supernatant of each well was replaced by 0.5 mg mL^{-1} MTT in culture medium, and the cells were incubated for 2 h, in the same conditions. The MTT solution was then replaced by hydrogen chloride-2-propanol solution 4 %, to lyse cells and to solubilize the formazan crystals. After 5 min at room temperature, the absorbance was read on a microplate reader (Spectra SLT, Tecan, Switzerland) at 540 nm. Cell viability was calculated by comparing the sample absorbance (A_{sample}) to the negative control absorbance ($A_{control}$), which was by definition 100 %, according to the equation $(A_{sample}/A_{control}) \times 100$ [28]. Data were expressed as mean ± standard deviation (SD) of three wells ($n = 3$). Statistical significance of CCPMs concentration changes in cell viability relatively to the negative control

were evaluated by one-way ANOVA (Microsoft Office Excel 2007). Tukey's HSD (honestly significant differences) test was used as a post hoc method to identify the significantly different means among CCPMs concentrations samples. Moreover, the toxic effect of CCPMs on cells viability was qualitatively observed by optical microscopy (Eclipse TE-300, Nikon, Japan). All the samples submitted to the cytotoxicity experiment were previously sterilized by exposure to UV light.

Results and discussion

Evaluation of CCPMs preparation procedure

In this study, CCPMs were prepared by an improved water-in-oil emulsion technique. Recently, other works were published where microparticles based on a similar polymeric matrix were prepared but using different preparation protocols [29–31], therefore, leading to microparticles with different characteristics. The improvements now presented aimed to prepare microsized particles of spherical and with smooth surface.

In our first attempts to produce glutaraldehyde cross-linked chitosan–PVA microparticles by water-in-oil emulsion, we used dichloromethane as organic phase [18]. However, we aspired to reduce toxic solvents in preparing microparticles. In this way, we decided to use vegetable oils as organic phase. Moreover, as the organic phase viscosity is one of the parameters which influence the size of microparticles produced by emulsion technique [32], it would be advantageous to use more viscous continuous phase to control microparticles size. Commercially, several vegetable oils are available with different viscosities [33]. This work was mainly focused in the use of corn oil as organic phase. However, sunflower and soya been oils were also tested leading to similar results (data not shown).

In order to form spherical polymer droplets, water was removed from polymeric phase, diffusing into the vegetable oil phase and then evaporating at the oil/air interface [34]. By adding methanol to the polymeric solution, water removal was improved and, thus, the formation of spherical polymer droplets in vegetable oil [25, 35]. Although the use of a suitable stabilizer is pointed out in most preparation procedures of microparticles by emulsion, when using a vegetable oil that step can be avoided [32].

In this water-in-oil emulsion technique, two steps could be distinguished: (i) production of two-phase systems involving the dispersion of a chitosan and PVA solution in vegetable oil, leading to the formation of small droplets of polymeric solution in immiscible liquid; (ii) conversion of initially formed polymer droplets into the corresponding microparticles, as a result of gradual hardening of the droplets by cross-linking, due to the addition of glutaraldehyde. Water-soluble polymers with hydroxyl groups, as PVA, as well as amine-containing groups, like chitosan, can be cross-linked with glutaraldehyde. Glutaraldehyde in the organic phase induced the reaction between aldehyde groups and amino or hydroxyl groups at the surface of the polymer goblets. Consequently, there should be a high cross-linking density near the surface of the CCPMs, possibly, accompanied by

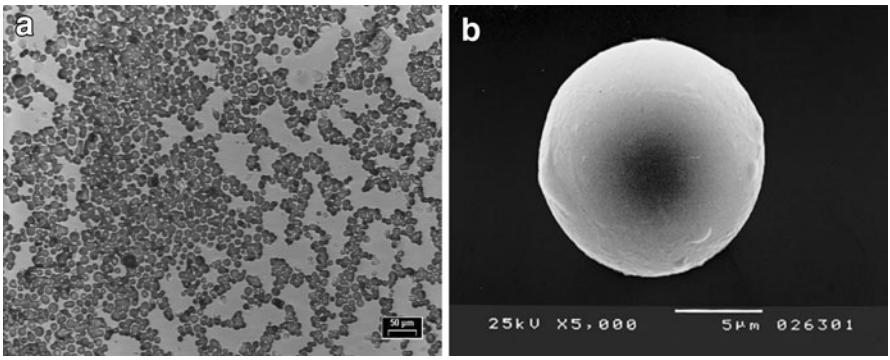


Fig. 1 Photographs of CCPMs acquired by **a** optical microscopy (×10) and **b** SEM (×5,000)

monoreacted dialdehyde at the surface. The resulting of monofunctional glutaraldehyde capping of chitosan and PVA at the CCPMs surface could, later on, facilitate a variety of chemical modifications including enzyme, antibody, or other protein ligand or even the tissue immobilization [23]. In this study, glutaraldehyde was added to the organic phase as solution saturated in toluene, improving its solubility in that phase and, therefore, enhancing cross-linking reaction.

Morphological characterization

The morphology of the CCPMs produced was investigated by optical microscopy and SEM. Examination of the CCPMs by optical microscopy revealed the formation of spherical particles, with regular shape, well-formed, without aggregation and, apparently, homogeneously distributed (Fig. 1a). Figure 1b shows a representative SEM photograph of the CCPMs surface. These CCPMs have smooth and uniform surface indicating the optimization of solvent evaporation rate during polymer droplets formation process, as described above. Besides, as the hardening of chitosan and PVA matrix was performed, with constant stirring at high speed, friction of vegetable oil with the CCPMs might have resulted in spherical geometry of these same particles, turning it smooth and with compact surface. The SEM observation also showed the nonexistence of pores on the surface of the CCPMs neither evidence of collapsed particles.

Particle size and particle size distribution

Particle size is a key property in certain processes related with the surface area, as degradation time [36]. While improving this procedure, speed of stirring was taken into account because it strongly determines particle size [37]. In fact, increasing speed of stirring leaded to smaller particles as a more efficient dispersion of polymeric phase in the organic one was achieved (data not shown). Moreover, it is known that the smaller the radius of a sphere, the larger is its surface area/volume ratio. By producing smaller microparticles, a more efficient release or adsorption system would be achieved, for instance.

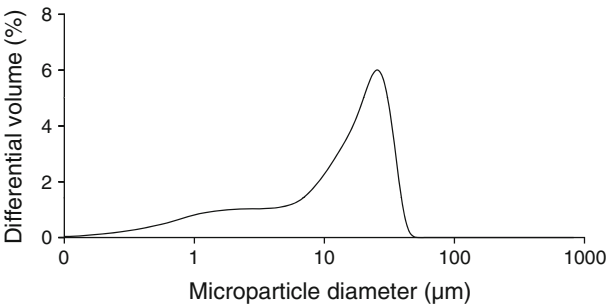


Fig. 2 Particle size distribution of CCPMs

The particle size distribution of CCPMs is shown in Fig. 2. CCPMs had particle size inferior to 49 μm in diameter and mean particle size was 16 ± 11 μm. Statistical analysis of CCPMs showed that 10 % of CCPMs sample population was above 32 μm diameter, 50 % was above 16 μm and 90 % was above 1 μm. Concerning skewness, which is a measure of the asymmetry of the frequency distribution, the value obtained was positive (0.289), meaning that the distribution was deviated to smaller size particles.

Chemical analysis of CCPMs polymeric matrix

FTIR spectroscopy was used to verify the structural modifications upon cross-linked reaction during preparation of CCPMs. Figure 3 shows the FTIR spectra of chitosan, PVA, and CCPMs. The FTIR spectrum of chitosan (spectrum a) showed a broad band at 3,285 cm^{-1} due to O–H and amine N–H stretching. The peak at 2,867 cm^{-1} was assigned to C–H stretch vibration. In addition to the peak at 1,586 cm^{-1}, assigned to amide II, there were also peaks at 1,639 and 1,320 cm^{-1}, characteristic of chitin and chitosan, which have been reported as amide I and III bands, respectively. The peak at 1,373 cm^{-1} was assigned to CH_3 deformation, whereas the peak at 1,420 cm^{-1} was assigned O–H and C–H deformation. A significant peak around 1,027 cm^{-1} is corresponding to C–O bending, typical of saccharide structure [38]. Regarding the spectrum of PVA (spectrum b), the characteristic absorption band at 3,212 cm^{-1} was attributed to stretching of O–H group; bands at 2,914 cm^{-1} and at 1,238 and 844 cm^{-1} were assigned to C–H stretching and bending, respectively, whereas the band at 1,090 cm^{-1} was due to C–O stretching. The band observed at 1,732 cm^{-1} was characteristic of carbonyl group of PVAc. In fact, as PVA is prepared from PVAc, a certain amount of PVAc may still exist in PVA batch [39]. Oxidation of PVA occurred during the manufacturing and processing [40] can also explain the presence of PVAc. In the spectrum of CCPMs (spectrum c), a new peak was found at 1,644 cm^{-1}, which can be assigned to the C=N stretching vibration from the imine bond [13] formed by the reaction between amino groups of chitosan and aldehyde groups of glutaraldehyde. At 1,063 cm^{-1}, another new peak was observed, which was assigned to the acetal bond [41], due to the reaction of glutaraldehyde with the hydroxyl group of PVA.

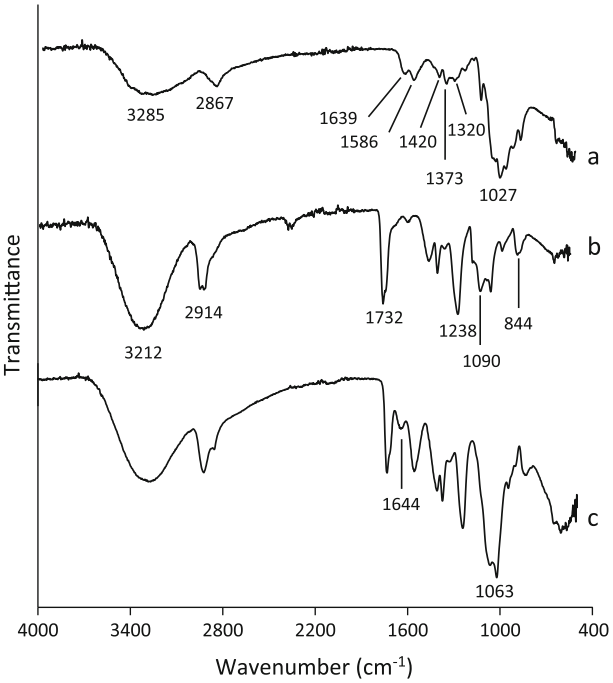


Fig. 3 Representative FTIR spectra of (*a*) chitosan, (*b*) PVA, and (*c*) CCPMs

CCPMs cytotoxicity

Cytotoxicity evaluation is on the first lane for screening biocompatibility or biological safety of materials. In general, accepted cytotoxic effects of materials are: cell death, loss of membrane integrity, reduced cell adhesion, altered cell morphology, reduced cell proliferation, and reduced biosynthetic activity [42]. There are several rapid, standardized, sensitive, and inexpensive tests to determine if a material contains significant quantities of biologically toxic extractables. The MTT cytotoxicity test is widely used for this purpose. Yellow water-soluble MTT is reduced by mitochondrial succinate dehydrogenase in viable cells to a blue–violet insoluble formazan. The number of viable cells is correlated to the color intensity, which is quantified using a spectrophotometer, after dissolving the formazan in acidic alcohol [43]. In this study, Wistar mouse peritoneal macrophages viability was measured after 20 h of incubation with 0.2, 2.0, and 10.0 % (v/v) CCPMs in culture medium (Fig. 4). According to one-way ANOVA, the cell viability of the negative control was significantly different from the one of the CCPMs concentrations of 2.0 and 10.0 % (v/v) samples ($p < 0.01$). Differences between cell viability of CCPMs concentration of 0.2 % (v/v) and of 2.0 % (v/v) ($p < 0.05$) and 10.0 % (v/v) ($p < 0.01$) were also identified as statistically significant. As cell viability was >70 % of the negative control in all tested CCPMs concentrations, according to the guidelines of ISO 10993-5 for biological evaluation of medical

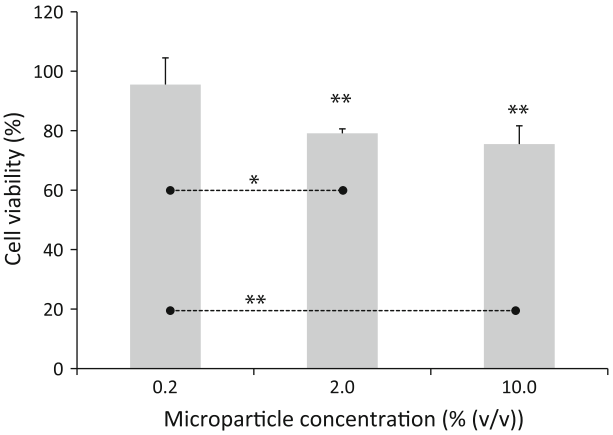


Fig. 4 CCPMs cell toxicity assessment performed by MTT assay on Wistar mouse peritoneal macrophages. Percentage of viable cells relative to the negative control after 20-h incubation with CCPMs at different concentrations. Data are represented as the mean ± SD ($n = 3$). *$p < 0.05$; **$p < 0.01$ (one-way ANOVA and Tukey's HSD test)

materials, we assumed that CCPMs matrices were able to preserve viability of cells, having, thus, no cytotoxic potential. Figure 5 shows optical micrographs of negative control and 2.0 % (v/v) CCPMs 20-h incubation, before and after the addition of MTT. The purple color due to formazan crystals inside cells (Fig. 5b, d) suggested that incubation with CCPMs did not affect cell viability.

The use of glutaraldehyde as cross-linking agent in the preparation of materials, in particular biomaterials, has been cause of concern as it is related to inflammatory reactions, cytotoxicity, calcification, and lack of endothelialization. However, reports of cytotoxic effects of glutaraldehyde were, in general, due to the use of a high glutaraldehyde concentration [14]. In this study, despite the use of toxic glutaraldehyde, results revealed no adverse effect on cells viability suggesting that its toxicity was likely minimized by the cross-linking reaction underwent in the preparation of CCPMs by the present procedure [44].

Conclusion

CCPMs were prepared by an improved and more environmentally friendly water-in-oil emulsion method. Results showed the formation of particles with good spherical shape, nonagglomerated, with smooth surface, and mean diameter of 16 μm. FTIR analysis suggested cross-linking reaction so that both imine and acetal groups were likely to occur during CCPMs preparation. Despite the use of glutaraldehyde as cross-linking agent, CCPMs proved to be noncytotoxic. We believe that this procedure can be followed straightforwardly in the preparation of CCPMs for a broad range of biotechnological applications.

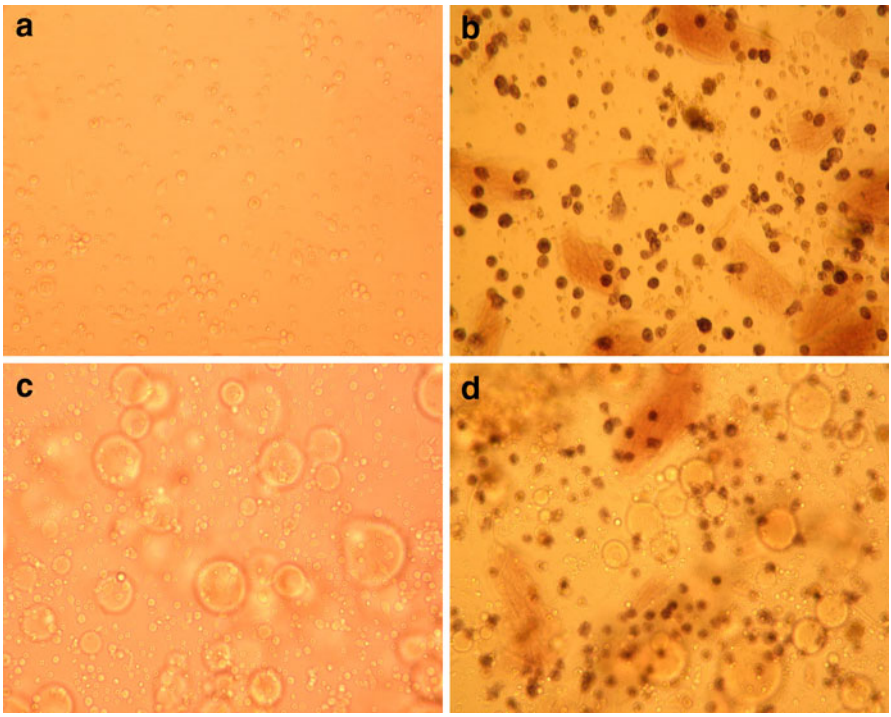


Fig. 5 Representative optical microphotographs of Wistar mouse peritoneal macrophages 20-h incubated in **a** culture medium (negative control), before addition of MTT, **b** culture medium (negative control), after addition of MTT, **c** CCPMs 2.0 % (v/v) in culture medium, before addition of MTT, and **d** CCPMs 2 % (v/v) in culture medium, after addition of MTT

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