In vitro cytotoxicity testing of chitosan-containing polyelectrolyte complexes

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Two chitosan-containing polyelectrolyte complexes, chitosan-poly(acrylic acid) and chitosan-poly(styrenesulphonate), were synthesized by polymerizing acrylic acid and sodium styrenesulphonate in the presence of chitosan and chitosan hydrochloride, respectively. The complexes were studied by optical microscopy and tested for cytotoxicity by the Neutral Red uptake, Kenacid Blue R-Binding and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assays. The optical microscopy confirmed the differences in crystallinity and structure already found for the two polycomplexes by other characterization techniques. The cytoxicity tests showed different influences on the cell activity by the extracts of the two polyelectrolyte complexes. Such results were discussed and correlated to the different structures of the two materials. © *1998 Chapman & Hall*

1. Introduction

In the literature, several works concerning the peculiar properties of polyelectrolyte complexes useful for biomedical applications are reported [1, 2]. Recently, we prepared poly(allylammonium acrylate) complexes (PAAC), having physicochemical and biological properties suggesting possible uses as polymeric matrices for drug release [3, 4]. Subsequently, we reported also on the synthesis and the physicochemical characterization of new polyelectrolyte complexes based on chitosan (CHI), namely chitosan-poly(acrylic acid) (CHI-PAA) and chitosan-poly(styrenesulphonate) (CHI-PSS) [5]. In the biomaterial field, chitosan-based complexes are well known by their application for the immobilization of enzymes, the micro-encapsulation of cells and the controlled drug release [6, 7].

In this paper, we report some cytotoxicity tests, as well as some water sorption experiments, made on CHI-PAA and CHI-PSS complexes as preliminary tests for their possible use as drug-delivery systems.

2. Materials and methods

2.1. Polyelectrolyte complex synthesis and characterization

The polyelectrolyte complexes CHI-PAA and CHI-PSS have been synthesized by radically polymerizing acrylic acid and sodium styrenesulphonate in the presence of CHI and CHI hydrochloride, respectively, following the procedure already reported [5]. The acrylic to CHI ([AA]/[CHI]) and styrenesulphonate to CHI ([SS]/[CHI]) repetitive unit ratios were 4 and 6, respectively. Optical microscopy (OM) was carried out by a Leitz Ortholux II POL-BK instrument on finely powdered samples of the materials.

2.2. Cytotoxicity tests

The cytotoxicity of both materials was tested by the Neutral Red uptake (NR), Kenacid Blue R-Binding (KB) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays according to the protocols already followed [4].

2.3. Water sorption tests

To measure the water sorption capacity of the polyelectrolyte complexes, weighed samples were sintered into tablets by compression under vacuum; the tablets were dipped in Dulbecco's PBS (pH = 7.4) and in glycine/NaOH (pH = 8.95) buffers at 37 °C; at regular time intervals the tablets were blotted with a paper towel to remove excess solution and then weighed.

3. Results

Fig. 1 shows an optical micrograph of the CHI-PAA complex; the presence of a crystalline structure in the powder granules is well visible. Fig. 2 shows optical micrographs of the CHI-PSS complex (a) and of the

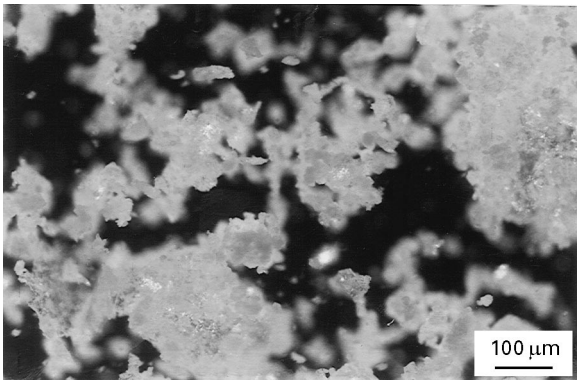

Figure 1 Polarized light optical micrograph of a CHI-PAA ([AA]/[CHI] = 4) complex.

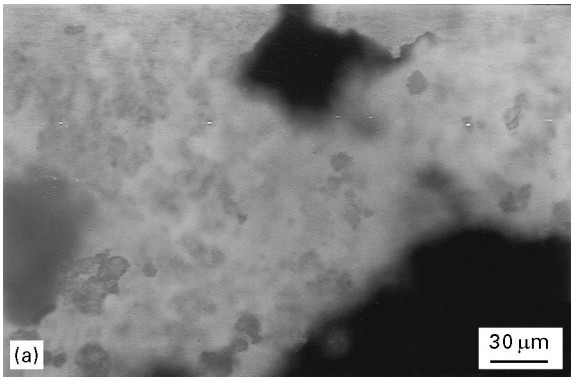

Figure 2 Polarized light optical micrographs of a CHI-PSS ([SS]/[CHI] = 6) complex (a) and of a corresponding "mixture" (b).

corresponding "mixture", obtained by coprecipitating the solutions of the preformed parent polymers (b); the crystalline structure is less visible than in CHI-PAA, and no difference can be seen between the complex and the "mixture". Fig. 3 shows an optical micrograph of some PAAC granules: each granule has a crystalline shape.

Because our interest involves the use of the above complexes as polymeric matrices for drug delivery systems, cytotoxicity tests were carried out by measuring the lysosomial and mitochondrial functional integrity and the proliferative capacity of 3T3 fibroblast cells. The results of the acute cytotoxicity tests after 48 h incubation show different behaviour. In the NR assay (Fig. 4), the lysosomial activity of both materials is favourably influenced by the extracts, to a greater

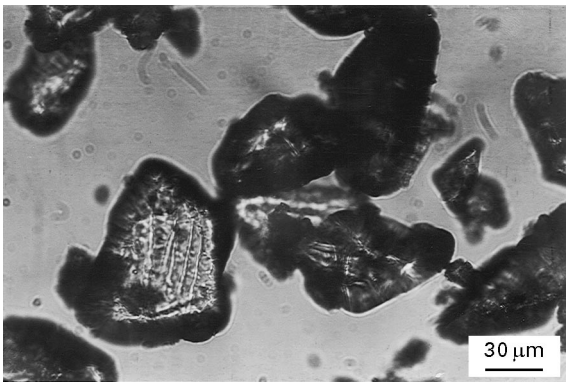

Figure 3 Non-polarized light optical micrograph of PAAC.

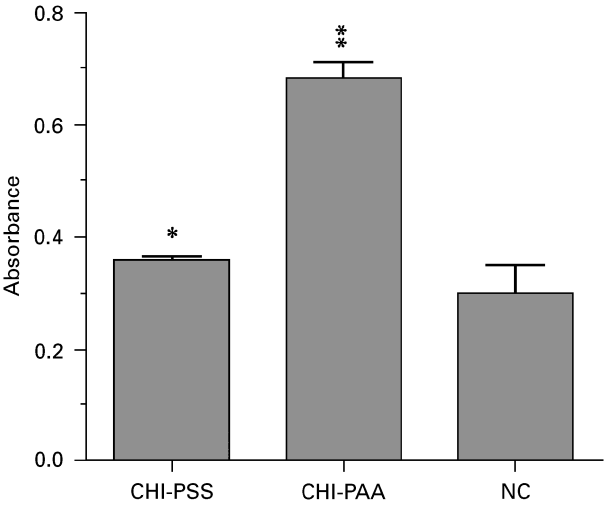

Figure 4 Column diagrams of the NR uptake results, after 48 h incubation (37 °C in a 5% CO_2 atmosphere) with the extracts of CHI-PAA ([AA]/[CHI] = 4) and CHI-PSS ([SS]/[CHI] = 6); NC: negative control (no extract added). The data are the mean ± SD, $n = 6$. Paired t-test, significance level: $*p \leq 0.05$, $**p \leq 0.01$. The statistical significance is referred to the NC.

extent for CHI-PAA than for CHI-PSS. In the KB assay (Fig. 5), the cell proliferation is depressed by CHI-PSS and greatly enhanced by CHI-PAA. In the MTT assay (Fig. 6), the mitochondrial activity is negatively affected by the extracts, as compared with the negative control, more for CHI-PSS than for CHI-PAA.

In view of a use of these new complexes as drug-releasing materials, we tried to carry out water sorption tests in the presence of both Dulbecco's PBS (pH = 7.4) and glycine/NaOH (pH = 8.95) buffers. After 30 min dipping in both buffers, the CHI-PAA tablets begin to disintegrate; disintegration of the CHI-PSS tablets occurs after only 15 min dipping.

4. Discussion

The results of the cytotoxicity tests of the two CHI-containing polyelectrolyte complexes (see Figs 4–6), are different from each other, and both different from those of PAAC [4], thus may be explained by the different structures of the complexes. In a preceding work [3] we found that PAAC is a very "tight"

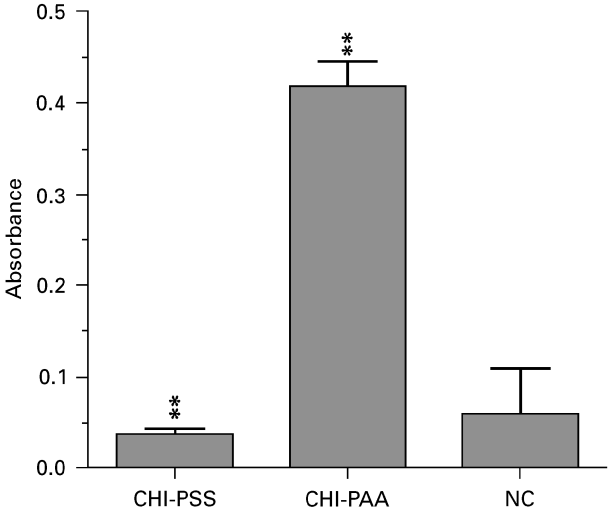

Figure 5 Column diagrams of the KB assay results, after 72 h incubation (37 °C in a 5% CO_2 atmosphere) with the extracts of CHI-PAA ([AA]/[CHI] = 4) and CHI-PSS ([SS]/[CHI] = 6); NC: negative control (no extract added). The data are the mean ± SD, $n = 6$. Paired t-test, significance level: $^{**}p \leq 0.01$, The statistical significance is referred to the NC.

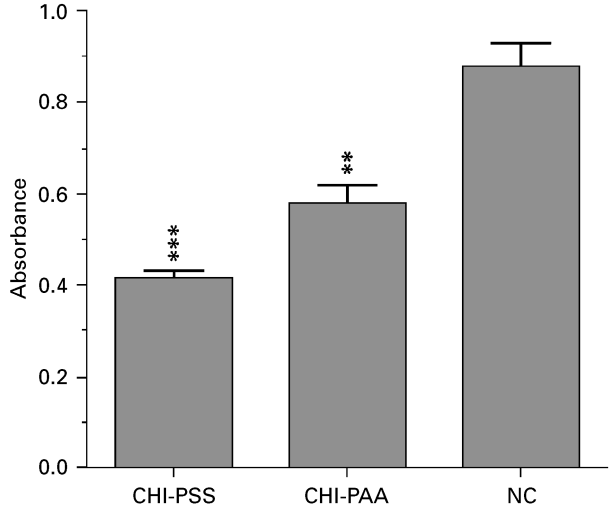

Figure 6 Column diagrams of the MTT assay results, after 72 h incubation (37 °C in a 5% CO_2 atmosphere) with the extracts of CHI-PAA ([AA]/[CHI] = 4) and CHI-PSS ([SS]/[CHI] = 6); NC: negative control (no extract added). The data are the mean ± SD, $n = 6$. Paired t-test, significance level: $^{**}p \leq 0.01$, $^{***}p < 0.001$. The statistical significance is referred to the NC.

complex, formed by a type I ("zip") mechanism. Of the two CHI-containing polycomplexes [5], CHI-PAA is a quite ordered one, having a likely structure similar to that shown in Fig. 7; on the contrary CHI-PSS, which is formed by a type II ("pick up") mechanism, is not distinguishable from the polysalt precipitated by mixing the preformed parent polymers, with a "scrambled egg" [8] structure.

The different structures of these polyelectrolyte complexes are also confirmed by the OM results shown in Figs 1–3. The sharp crystallinity of PAAC (see Fig. 3) agrees well with its very "tight" structure. The difference between CHI-PAA (see Fig. 1) and

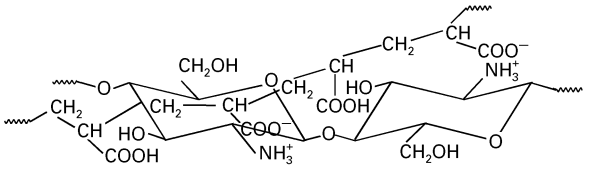

Figure 7 Most likely structure of a CHI-PAA polyelectrolyte complex.

CHI-PSS (see Fig. 2a) structures, as well as the absence of any substantial difference between the latter and the corresponding "mixture" (see Fig. 2b), is also evident in the optical micrographs.

Because of these different structures, PAAC has the lowest solubility in PBS, so that the extracts contain a very low concentration of its parent polymers, that is of substances able to influence the cellular activity; a greater quantity is released by CHI-PAA, which disintegrates in PBS within 30 min, and the highest quantity by CHI-PSS, which disintegrates in PBS within 15 min. Although the disintegration times of the polysalt sintered tablets cannot be assumed as a measure of their solubility, these two properties are somehow correlated.

In a previous paper [4] we showed that PAAC did not influence the results of the same cytotoxicity tests, as compared with the negative control. On the contrary, our present results indicate that there is an influence of the material's extracts on the 3T3 cell metabolic responses measured and that this influence is a positive one only in the case of CHI-PAA. In the case of MTT assay, the negative influence of both polyelectrolyte complexes on mitochondrial activity is less strong in the case of CHI-PAA. A possible explanation is that the structure of the more stable and less soluble polysalt CHI-PAA is favourable to the cells.

From the NR and MTT assays, the results can be interpreted by supposing different influences on the cell activity by the substances released by the polyelectrolyte complexes, i.e. chitosan cation ($CHIH^{n+}$), polyacrylate anion (PA^{n-}) and poly(styrenesulphonate) anion (PSS^{n-}). The results shown in Figs 4 and 6 lead us to suppose that $CHIH^{n+}$ influences favourably the lysosomial activity, but not the mitochondrial activity. Conversely, the polyanions seem to influence negatively both NR and MTT; the less negative influence of PA^{n-} may be explained by its concentration in the extracts, lower than that of PSS^{n-}.

From the KB assay, the data in Fig. 5 favour the hypothesis that the cell proliferation is influenced positively by PA^{n-}, and negatively by PSS^{n-}.

Such different influences of the polyions on the cytotoxicity tests are still hypothetical, and must be checked by specific experiments.

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