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Cytotoxic evaluation of injectable cyclodextrin nanoparticles

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Abstract

Nanoparticles were prepared using β-CDC6, which is an amphiphilic β-cyclodextrin derivative modified on the secondary face with 6C aliphatic esters. A nanoprecipitation technique was used to prepare the blank nanoparticles without any surfactant and nanoparticles containing Pluronic F68 as surfactant in a concentration range of 0.1 to 1%. Nanoparticle formulations were characterized by particle size distribution and zeta potential measurements. Entrapment efficiency and in-vitro release profiles were determined and the cytotoxicity of these injectable nanospheres was evaluated against mouse fibroblast L929 cell line and human polymorphonuclear cells by methlythiazolyltetrazolium assay. As far as particle size and zeta potential are concerned, there is a relationship between surfactant presence and nanoparticle characteristics. However, these effects are not significant. It was also found that surfactant presence has no effect on model drug nimodipine encapsulation but accelerates the in-vitro release of the drug. Cell culture studies on mouse fibroblasts and human polymorphonuclear cells revealed a concentration-dependent cytotoxicity more pronounced in fibroblast cells. This led to the conclusion that the use of surfactants in injectable nanoparticles prepared from amphiphilic β-cyclodextrins may lead to altered in-vitro properties and impaired safety for the drug delivery system.

Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides obtained by the enzymatic degradation of starch by cyclodextrin glucosyl transferase enzyme. CDs are of pharmaceutical interest mainly because they are used to formulate drugs with poor aqueous solubility, physical or chemical instability or adverse effects (Loftsson & Brewster 1996; Szejtli 1998). They are capable of forming inclusion complexes with a variety of guest molecules owing to their special structure, which consists of a hydrophilic external surface and a hydrophobic cavity lined with protons. Natural and synthetic CDs have the ability to form inclusion complexes partially or totally in their hydrophobic cavity, which helps to mask the included drug's physicochemical properties. Different chemical modifications have been carried out on natural CDs to obtain a wide variety of CD derivatives with altered solubility, release and absorption properties (Albers & Müller 1995).

Amphiphilic β-CDs have been synthesized and used for pharmaceutical applications since the last decade after it was demonstrated that these molecules have the ability to form supramolecular aggregates in the form of nanoparticles (Zhang & Parrot-Lopez 1992). They form stable nanospheres and nanocapsules with and without the presence of a series of surfactants including Pluronic F68 (PF68), Polysorbate 80 and Span 80 (Skiba et al 1996; Duchene et al 1999). One of the major disadvantages of CDs in the pharmaceutical field is the haemolysis they cause on injection, along with their nephrotoxicity (Frank et al 1976; Irie & Uekama 1997). Amphiphilic β-CDs recently modified on the primary or secondary faces, however, have been demonstrated to be non-haemolytic against whole blood and erythrocyte suspensions (Memişoğlu et al 2002a, 2003a). β-CDC6 modified on the secondary face with 6C aliphatic esters was used in this study as an amphiphilic β-CD type.

Most injectable nanoparticles have a polymeric structure of natural, semi-synthetic or synthetic origin. They are generally prepared with the aid of surfactants since the polymer

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itself does not generally have the ability to form stable nanostructures (Couvreur 1996, 2002). CD-based nanoparticles do not require the presence of a surfactant, thus they are believed to reduce the potential of overall toxicity for the drug carrier system (Wouessidjewe et al 1996; Lemos-Senna et al 1998).

The objective of this study was to evaluate the cytotoxicity of β-CDC6 nanoparticles with and without the presence of PF68 in order to determine the possibility of presenting an injectable nanosized drug carrier system that can be formulated without any surfactants as an alternative to injectable polymeric nanoparticles that require the presence of surfactants in considerable amounts. For this reason, PF68 was selected as it is the most frequently used surfactant in nanoparticle formulations in the pharmaceutical field. In-vitro properties such as particle size distribution, zeta potential, drug loading and in-vitro release profiles were also assessed in formulations prepared with and without surfactants. Nimodipine, a water-insoluble (aq. sol. 2.3 μg mL^{-1}) calcium channel antagonist with therapeutic indications for cerebrovascular spasm, stroke and migraine, was used as a model drug in this study (Gelmers 1985; Langley & Sorkin 1989). Cytotoxicity studies were performed against L929 fibroblast cells and human polymorphonuclear (PMN) cells.

Materials and Methods

Materials

β-CDC6 is an amphiphilic β-CD derivative selectively peracylated on its secondary hydroxyls with 6C aliphatic esters. This product has been synthesized, purified and characterized as reported previously (Memişoğlu et al 2002b). PF68 was obtained from ICI Surfactants (Clamart, France). Miglyol 812 (used as the oil phase in the nanocapsules) was obtained from Condea Chemie, Witten, Germany. Acetone and all other reagents were of HPLC grade and were used without further purification. L929 mouse fibroblast cell line was obtained from the American Type Tissue Cell Culture Collection (ATCC) and PMN cells were isolated from blood samples drawn from healthy volunteers.

Preparation and characterization of nanospheres/nanocapsules

β-CDC6 nanoparticles were prepared using the nanoprecipitation technique (Fessi et al 1988) modified for amphiphilic CDs (Skiba et al 1992a, b). Nimodipine-loaded nanospheres and nanocapsules were prepared directly from drug:CD inclusion complexes as described previously (Memişoğlu 2003a). Mean diameter, polydispersity index and zeta potential values were determined for different nanoparticle formulations. The amount of encapsulated drug in the β-CDC6 nanoparticles was determined according to the procedure previously reported (Memişoğlu et al 2003a, b) with quantification of nimodipine using a UV spectrophotometric method at 354 nm (Hu et al 2003). Drug loading was expressed in terms of entrapped drug quantity, which is the determined drug quantity in the nanoparticle dispersion after elimination of unbound drug with centrifugation and entrapment efficiency (entrapped drug (μg) per unit CD (mg)). Nimodipine in-vitro release was determined in isotonic phosphate buffer solution (PBS) at 37 °C with 1% Polysorbate 80 providing sink conditions in a thermostatted shaker bath system.

Cytotoxicity of blank nanospheres/nanocapsules

L929 mouse fibroblasts (ATCC) were cultured in 25 cm^2 culture flasks containing RPMI 1640 supplemented with heat inactivated 10% fetal bovine serum, 2 mM L-glutamine, 100 units mL^{-1} penicillin G and 100 μg mL^{-1} streptomycin at 37 °C in a humidified incubator containing 5% CO_2. Confluent cell monolayers were trypsinized and cells in the exponentially growing phase were used in the cytotoxicity experiments.

Healthy donor leukocytes (PMN cells) were isolated using Histopaque 1077 (Sigma Chemical Co., St Louis, MO, USA) density gradient and cultured immediately at a concentration of 2×10^6 cells mL^{-1} in the same culture conditions. Nanocapsules (0.4 mM) and nanospheres (0.4 mM) were diluted as indicated in the relevant figures.

The methlythiazolyltetrazolium (MTT) assay was used to evaluate cell viability (Campling et al 1988; Hansen et al 1989). Briefly, 50 μL cell suspensions containing 2×10^5 leukocytes or 10^3 L929 cells were seeded in 96-well plates (Costar, Cambridge, MA) and 50 μL of diluted material was added to each well. Flat-bottomed 96-well plates were utilized for L929 cells whereas round-bottomed plates were used for leukocytes. After 72 h of incubation, 25 μL of MTT solution (1 mg mL^{-1} final concentration; Sigma Chemical Co., St Louis, MO, USA) was added to each well and the plates were incubated for a further 4 h. The formazan precipitate was solubilized by adding 80 μL lysing buffer (pH = 4.7) composed of 23% sodium dodecyl sulfate dissolved in a solution of 45% N, N-dimethylformamide. After overnight incubation at 37 °C, the optical density (OD) was read at 570 nm using a microplate reader (Spectramax Plus, Molecular Devices, Sunnyvale, CA, USA). Cells incubated in culture medium alone served as a control for cell viability (non-treated wells). Cell viability (%) was calculated as (OD of treated wells/OD of non-treated cells) $\times$ 100.

Statistics

Kruskall–Wallis analysis of variance was used to analyse the drug encapsulation of different formulations. The in-vitro release data were analysed by two-way analysis of variance (ANOVA) for repeated measures test and data at specific time intervals were analysed by Tukey's test. Cytotoxicity data were analysed by two-way ANOVA for repeated measures using the ANOVREP program.

Results and Discussion

The objective of this study was to evaluate the cytotoxic properties of β-CDC6 nanospheres and nanocapsules on injection. β-CDC6 nanospheres are matrix-type drug delivery systems consisting of a spherical network formed by the interfacial deposition of the macromolecule β-CDC6 at the acetone/water interface. Nanocapsules, however, are membrane-type systems with an oil core surrounded by the amphiphilic β-CD aligned at the oil/water interface.

To evaluate the cytotoxicity of β-CDC6 nanoparticles manufactured with or without surfactants, PF68 was incorporated in the formulations within a concentration range of 0.1 to 1%, covering the most frequently used concentrations of this widely used surfactant in polymeric nanoparticles. The study was therefore designed to evaluate the cytotoxicity of β-CDC6 nanospheres and nanocapsules, and the effect of formulation constituents on cell viability.

The effect of surfactant presence on nanoparticle in-vitro characteristics was observed together with cytotoxicity studies. Table 1 summarizes the effect of PF68 presence on mean diameter, polydispersity index (PI) and zeta potential. Mean diameter is reduced significantly for nanospheres when PF68 is included in the formulation. This effect is not as significant for nanocapsules mainly because nanocapsule size is limited to the oil droplet size formed during preparation. PI values are favourable (below 0.25) for all formulations and are not affected by the presence of PF68 or concentration. Zeta potential, on the other hand, is largely dependent on the presence of PF68, reducing the negative charge of β-CDC6 nanoparticles with its non-ionic nature. Drug loading is around 150 μg with an entrapment efficiency of 17% for the model drug nimodipine.

Figure 1 shows the in-vitro release profiles of nimodipine from blank or PF68 containing β-CDC6 nanoparticles. Nanoparticles released the model drug nimodipine completely in 6 h. However, when formulated with 0.5% PF68, complete release is observed in 3 h with a burst effect characterized by 70% release in approximately 15 min. The release mechanisms of drugs from CD complexes are governed by two different phenomena: liberation of the drug from the CD cavity and diffusion of the drug carrier system, which is either matrix or membrane type. Furthermore, release of drug from the complex is believed to be based on time-dependent mechanisms such as dissociation of drug on dilution and competitive displacement of drug from surrounding medium constituents (Stella et al 1999). In the literature there are various reports of surfactants forming inclusion complexes with CDs (Garcia et al 1990; Fenyvesi et al 1992; Mwakibete et al 1995) and the dissolution enhancing effect of surfactants on drug:CD inclusion complexes (Veiga & Ahsan 2000). Undoubtedly the solubilizing property of PF68 on the water-insoluble drug nimodipine also may accelerate its release from the nanoparticles, as in this study.

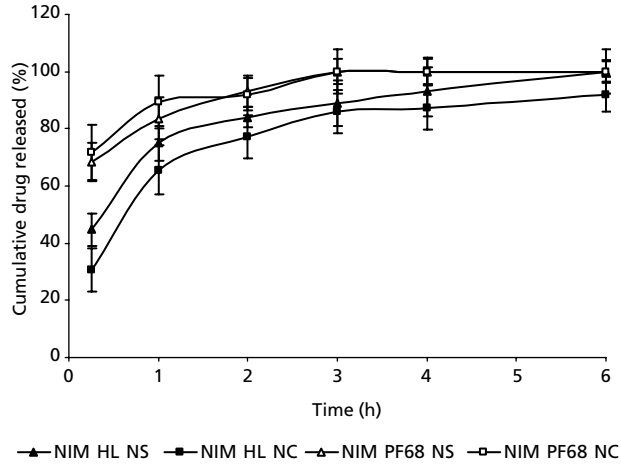

Figure 1 In-vitro release profiles of nimodipine-loaded nanoparticles with and without surfactants (n = 3, ± s.d.). NIM HL NS, nimodipine-loaded nanospheres; NIM HL NC, nimodipine-loaded nanocapsules; NIM PF68 NS: nimodipine-loaded nanospheres containing 0.5% PF68; NIM PF68 NC, nimodipine-loaded nanocapsules containing 0.5% PF68.

Table 1 Particle size distribution and zeta potential values of nanoparticle formulations (n = 3, ± s.d.)

Formulation	Mean diameter (nm) ± s.d.	PI	Zeta potential (mV) ± s.d.
Non-surfactant nanospheres	281 ± 89	0.033	−29.7 ± 0.9
0.1% PF68 nanospheres	199 ± 60	0.151	−5.4 ± 0.3
0.5% PF68 nanospheres	161 ± 53	0.207	−5.6 ± 0.19
1% PF68 nanospheres	169 ± 39	0.047	−13.4 ± 0.66
Non-surfactant nanocapsules	289 ± 63	0.053	−19 ± 0.36
0.1% PF68 nanocapsules	263 ± 62	0.066	−13.1 ± 0.22
0.5% PF68 nanocapsules	267 ± 63	0.072	−14.2 ± 0.28
1% PF68 nanocapsules	265 ± 55	0.091	−15.0 ± 0.56

β-CDC6 nanoparticles are designed as injectable drug delivery systems. They are expected not to have cytotoxic effects on injection. It is believed that many factors and parameters could be effective in the overall cytotoxicity of β-CDC6 nanoparticles, such as β-CDC6 itself, surfactant PF68 and its concentration used in the formulation, residual solvents or the oil phase used in the formulation (Miglyol 812).

In order to assess the cytotoxicity of β-CDC6 nanospheres and nanocapsules, mouse fibroblast cell line L929 and PMN cells isolated from healthy donor blood samples were used in this study. In all formulations and cell lines, lower dilutions led to total cell death and incomparable results with L929 cells. However, at high dilutions, it was possible to differentiate between blank formulations and formulations that contained PF68. A level of 0.1% PF68 seems to result in a similar cell viability profile to blank nanoparticles, but for formulations containing 0.5 and 1% PF68 it can be observed that cytotoxicity is concentration dependent and is influenced by the surfactant. This effect is observed for both nanospheres (Figure 2) and nanocapsules (Figure 3) against L929 cells. As a control,

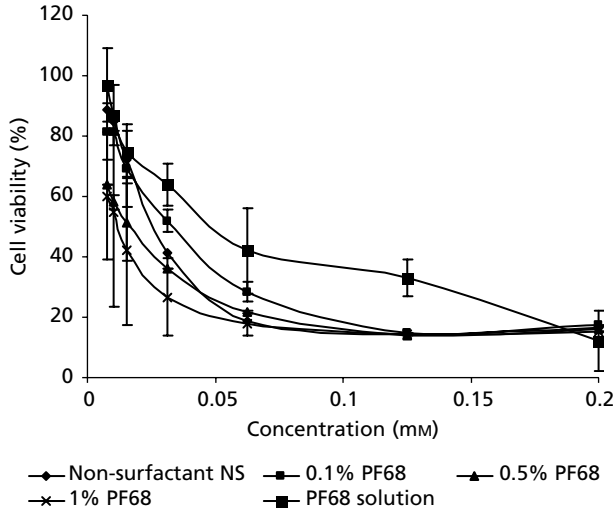

Figure 2 Cytotoxicity of nanosphere formulations on L929 mouse fibroblast cells.

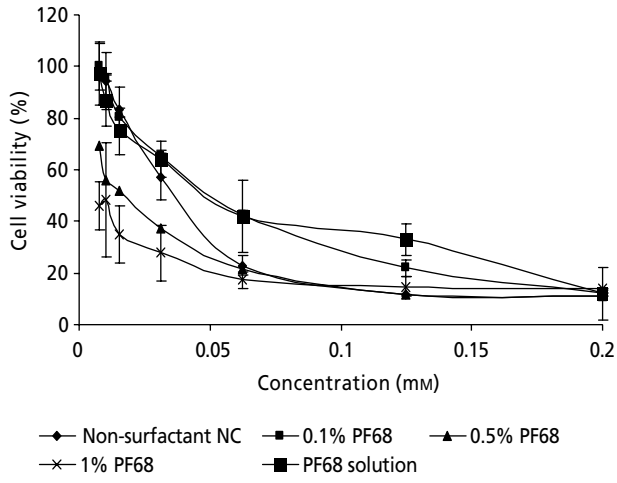

Figure 3 Cytotoxicity of nanocapsules on L929 mouse fibroblast cells.

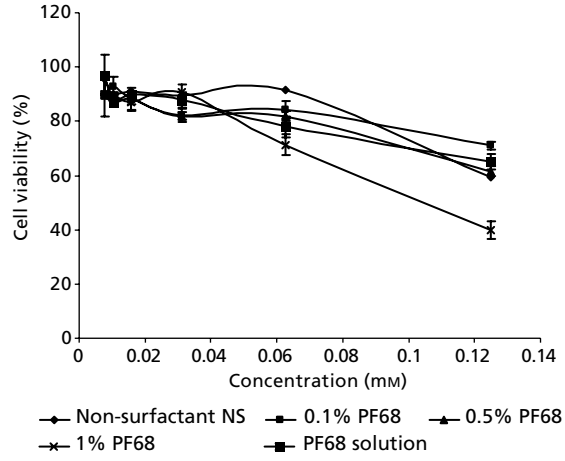

Figure 4 Cytotoxicity of nanospheres on human PMN cells.

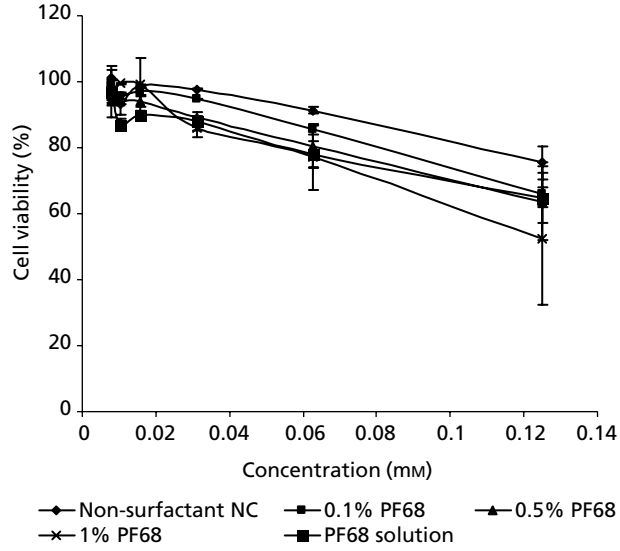

Figure 5 Cytotoxicity of nanocapsules on human PMN cells.

PF68 1% solution in deionized water and Miglyol 812 (5%) were also evaluated separately. Miglyol 812 had no effect on cell viability; however, PF68 solution exerted a concentration-dependent cytotoxicity very similar to PF68 containing nanoparticles. Figures 2 and 3 suggest that the main parameter affecting cytotoxicity is PF68 concentration; however, studies carried out with the control group (1% PF68 solution) indicate that the cytotoxicity of PF68 is potentiated when incorporated into nanoparticles. This may be attributed to the presence of β-CDC6 and residual solvent.

Figures 4 and 5 represent the viability of PMN cells for β-CDC6 nanospheres and nanocapsules. Cell viability is higher than for fibroblast cells, as expected, since there is a more highly populated cell line in these experiments. For PMN cells, the concentration-dependent cytotoxicity of PF68 nanoparticles is much higher than that of their non-surfactant analogues. A control experiment was performed with 1% PF68 aqueous solution. As seen in Figures 4 and 5, PF68 solution is as non-cytotoxic as blank nanoparticles. However, a concentration-dependent cytotoxicity increase is still observed, indicating that cytotoxicity against PMN cells is affected by the presence of β-CDC6 and residual solvent potentiating the cytotoxicity of PF68.

In general, the cytotoxicity order for both nanospheres and nanocapsules against fibroblasts and PMN cells is: non-surfactant < 0.1% PF68 < 0.5% PF68 < 1% PF68. These results suggest that the cytotoxicity of injectable β-CDC6 nanospheres and nanocapsules arises from various parameters, such as surfactant presence and concentration, the haemolytic and inclusion-forming capability of β-CDC6 with blood cell constituents and potential solvent residue.

Conclusion

From the in-vitro characterization studies and cell culture experiments, it can be concluded that regarding system safety and efficacy, formulation of β-CDC6 nanoparticles is possible avoiding the use of high concentrations of surfactant. The presence of a surfactant does not contribute to the injectability ensured by particle size or the stability of the drug carrier system indicated by zeta potential. It can also be suggested that amphiphilic β-CDC6 nanoparticles may present an alternative to other polymeric nanoparticles that require the presence of a surfactant to achieve an injectable size and to obtain stable systems. β-CDC6 nanoparticles do not exert a significant cytotoxicity against fibroblast and PMN cells. If required, 0.1% PF68 in the nanoparticle formulation gives similar cytotoxicity to non-surfactant nanospheres, with increasing cytotoxicity with increasing surfactant concentration.

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