Development of lyophilized spherical particles of poly(epsilon-caprolactone) and examination of their morphology, cytocompatibility and influence on the formation of reactive oxygen species

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A common limitation of using polymeric micro- and nanoparticles in long-term conservation is due to their poor physical and chemical stability. Freeze-drying is one of the most convenient methods that enable further reconstitution of micro- and nanoparticles for therapeutical use. Nevertheless, this process generates various stresses during freezing and desiccation steps. This paper underlines the combined outcomes of freeze drying method and physicochemical solvent/non-solvent approach to design biocompatible poly(epsilon-caprolactone) (PCL) nanospheres and evaluate influence of different cryoprotectants (glucose, saccharose, polyvinyl alcohol or polyglutamic acid) on the outcome of freeze-dried PCL particles. Samples were characterized using Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM) and dynamic light scattering method (DLS). In vitro studies used, include MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), testing cytotoxicity as the quality of being toxic to cells, and DCFH-DA assay (2',7'-dichlordihydrofluorescein-diacetate), testing the possible increase in ROS levels. It was found that cryoprotection with 1% glucose solution is an optimal for obtaining uniform, spherical but also biocompatible PCL nanoparticles for biomedical purposes.

Key words: PCL – Lyophilization – Cryoprotectants – Biocompatibility – Cytotoxicity.

During the last few decades, extensive work has been done to develop biodegradable polymers suitable for drug delivery studies and tissue engineering [1, 2]. In this way, most of the formulations, based on the incorporation of drugs to multiparticulate systems such as micro- or nanospheres, were prepared from aliphatic polyesters, such as poly(ε-caprolactone) (PCL), to ensure a specific drug targeting, by both the oral route and the parenteral route [3-6]. PCL, one of the most popular synthetic polymers, has been extensively investigated for applications as implantable or injectable biodegradable carrier for the controlled release of bioactive agents [7-9]. PCL is suitable for controlled drug delivery due to a high permeability to many drugs, excellent biocompatibility and its ability to be fully excreted from the body, once bioresorbed [9]. Biodegradation of PCL, compared to many other resorbable polymers, is slow, making it much more suitable for long-term degradation applications. PCL micro- and nanoparticles are promising drug delivery systems with obvious advantages, such as improving the overall therapeutic efficiency, prolonging biological activity, controlling the drug release rate and decreasing the administration frequency. Without occluding needles and capillaries, the size of nanospheres allows them to be administered intravenously via injection, unlike many other colloidal systems. However, the extensive application of micro- and nanospheres might be limited due to problems in maintaining the integrity of the liquid suspension for prolonged time period [10]. Besides the nontoxic properties and biodegradability, sufficient chemical and physical stability are crucial requirements for their industrial development [11, 12]. Freeze drying, also termed lyophilisation, is a process used to convert solutions of formulations into solids of sufficient stability for distribution and storage. This technique is a good method for conserving the integrity of particulate systems [13, 14]. However, freeze-drying is a complex process involving changes in temperature and therefore physical state of materials, as well as concentrations of different substances in the liquid environment, which all easily disturb the stability of nanoparticle dispersion [15]. Various stresses are generated during the process. Some of the stresses include freezing, desiccation and mechanical stresses. For example, the crystallization of ice exercises mechanical stress on nanoparticles leading to their destabilization. Also, too high concentration of particles in dispersion may induce their aggregation and irreversible fusion. Based on the literature, a number of components of the particles formulation, such as the type and concentration of cryoprotectant, the nature of surfactant, the chemical groups attached to the particles surface etc, may have significant influence on the resistance of particles to the different stresses during freeze-drying [15-17]. To protect nanoparticles from stresses and subsequent agglomeration, a cryoprotectant or lyoprotectant is generally used [16, 17]. The selection of a proper cryoprotectant is not straightforward. In some cases, increasing cryoprotectant concentration to a certain level can lead to destabilization of particulate systems [15]. Depending on the system of micro- and nanoparticles, freeze drying conditions and the characteristics of a cryoprotectant (thermal properties primarily), performance of the substance to decrease agglomeration can be erratic. Therefore, the type of a cryoprotectant must be wisely selected and its concentration must be optimized in order to ensure a maximum stabilization of nanoparticles.

The aim of this study was to produce PCL spherical particles, by physicochemical solvent/non-solvent method, and to evaluate influence of different cryoprotective excipients during freeze drying process. Sugars, such as glucose and saccharose, and polymers (polyvinyl alcohol (PVA) and polyglutamic acid (PGA)), were added to the formulation to test the effects on the outcome of freeze-dried poly(ε-caprolactone) micro- and nanospheres.

To date, the literature has described the synthesis of PCL materi-

als using an electrospinning [18, 19], dip-coating [20], ring-opening copolymerization [21, 22], solvent casting and melt blending methods [23]. These have provided materials in the forms of films, scaffolds, fibers, grafts or micron-sized particles. The literature has also describes the obtaining of camptothecin loaded PCL nanofibrous mats through one-step sol-gel electrospinning process [24], as well as the obtaining of PCL-based films co-loaded with 5-fluorouracil [20]. Our study thus reports on obtaining PCL nanospheres which represent an important system in the field of medicine, pharmacy and controlled drug delivery.

Samples were characterized using Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM) and dynamic light scattering method (DLS). *In vitro* studies used include MTT assay, testing cytotoxicity as the quality of being toxic to cells, and DCFH-DA assay, testing the possible increase in ROS levels, which may result in significant damage to cell structures and oxidative stress.

I. MATERIALS AND METHODS

1. Materials

Poly(ε-caprolactone) used in experiments was obtained from Lactel Absorbable Polymers (Birmingham, Alabama, United States) with average Mw of 50 kDa and intrinsic viscosity of 0.85 dL/g. Poly(L-glutamic acid) (PGA) with Mw = 20–40 kDa (99.9 % HPLC purity) was purchased from Guilin Peptide Technology Limited (China). Saccharose was a product from VWR BDH Prolabo, Belgium, while glucose, PVA, chloroform and ethanol were purchased from Superlab, Serbia. All reagents were of the analytic grade and were used as received without further purification. The following agents and chemicals used for the determination of cytotoxicity and the formation of ROS were obtained from Sigma Aldrich (St. Louis, United States): Eagle's Minimal Essential Medium, penicillin/streptomycin, L-glutamine, phosphate-buffered saline, trypsin, fetal bovine serum, non-essential amino-acid solution (100x), MTT, dimethyl sulfoxide, tert-butyl hydroperoxide and 2,7-dichlorofluorescein diacetate (DCFH-DA).

Cell culture

HepG2 cells were obtained from European Collection of Cell Cultures (ECACC). Cells were grown in Eagle Minimal Essential Medium containing 10 % fetal bovine serum, 1 % non-essential amino acid solution, 2 mM L-glutamine and 100 U/mL penicillin plus 100 μg/mL streptomycin at 37°C in humidified atmosphere and 5 % CO_2.

2. Freeze drying preparation of PCL particles without and with different cryoprotectants

PCL micro- and nanospheres were synthesized using physicochemical solvent/non-solvent method. Three hundred milligrams commercial granules of poly(ε-caprolactone) have been dissolved in 5 mL of organic solvent (chloroform). After approximately 1 h, ethanol (20 mL) has been rapidly added into the solvent mixture, followed by homogenisation on magnetic stirrer (500 rpm). At that instant, after the diffusion of solvent into non-slovent, PCL precipitates and the solution becomes whitish.

2.1. Selection of different cryoprotectants

PVA and PGA are commonly used as stabilizers. PVA is well known for its ability to form thin films. PGA is a highly anionic polymer, and with pH above its pK, carboxyl group is deprotonized, leaving negatively charged side chain of this polyelectrolyte. In this study, PVA and PGA will be evaluated for their ability to act as cryoprotectants. Carbohydrates are favoured as excipients because they can be easily vitrified during freezing. All excipients used are chemically innocuous.

Five millilitres of 1 % water solution of saccharose, poly(vinyl alcohol), poly(L-glutamic acid) or glucose was added to the formulation dropwise. The obtained suspensions were then homogenized on magnetic stirrer for less than 1 min at 500 rpm, and subsequently poured into a petri dish and put into the freezer overnight.

2.2. Freeze drying

After the freezing, method of lyophilisation was utilized at -55 °C and the pressure of 0.3 mbar. Main drying was performed for 8.5 h, and final drying for 30 min, using Freeze Dryer Christ Alpha 1-2/ LD plus.

3. Fourier transform infrared spectroscopy (FTIR)

FTIR measurements of the samples were carried out to identify the possible interactions between PCL and different cryoprotectants (PGA, PVA, glucose or sacharose), in terms of disrupting or creating new chemical bonds in the polymer structure. FTIR spectra of the samples were recorded in the range of 400-4000 cm^{-1} using a Carl Zeiss Specord 75 Spectrometer at 4 cm^{-1} resolution, using KBr pellet method.

4. Scanning electron microscopy (SEM)

Morphology imaging was realized using scanning electron microscop Jeol-JSM-646OLV, Japan. The energy of electrons was 10 to 50 KeV. The samples were coated with gold for conductivity improvements, using physical vapour deposition technique on 3ΔL-TEC SCD005 (Baltec SCD 005 sputter coater) - 30 nA current from a distance of 50 mm, during 180 s.

5. Particle size distribution (DLS)

The size distribution of PCL particles was determined by PSA Mastersizer 2000 (Malvern Instruments Ltd., United Kingdom). The size measurement range of this instrument is from 20 nm up to 2 mm. For the particle size measurements the powder was deagglomerated in an ultrasonic bath (frequency 40 kHz and power 50 W) for 15 min.

6. MTT assay

The cytotoxicity study was realized using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), according to Mossman *et al*. [25], with minor modifications [26]. Basis of this assay is the ability of dehydrogenase enzymes of intact mitochondria in living cells, to reduce MTT to insoluble formazan. The HepG2 cells were seeded onto 96-well microplates (Nunc, Naperville IL, United States) at a density of 40 000 cells/mL and incubated for 20 h at 37 °C to attach. The medium was then replaced by fresh complete medium containing 0, 0.0001, 0.001, 0.01, 0.1 and 1 % (v/v) of samples (PCL without cryoprotectants, PCL with 1 % PGA and PCL with 1 % glucose), and incubated for 24 h. In each experiment, a negative control (non-treated cells) and vehicle control (1 % emulsion) was included. MTT (final concentration 0.5 mg/mL) was then added, incubated for an additional 3 h, the medium with MTT was then removed and the formed formazan crystals dissolved in DMSO. The optical density (OD) was measured at 570 nm (reference filter 690 nm) using a microplate reading spectrofluorimeter (Synergy MX, Biotek, United States). Viability was determined by comparing the OD of the wells containing the treated cells with those of the non-treated cells. Five replicates per concentration point and three independent experiments were performed.

Statistical significance between treated groups and controls was determined by two tailed Student's t-test and $P<0.05$ was considered as statistically significant. Three independent experiments with five replicates were performed.

7. Determining intracellular reactive oxygen species formation – DCFH-DA assay

The formation of intracellular reactive oxygen species (ROS) was measured spectrophotometrically using a fluorescent probe, DCFH-DA as described by Osseni *et al*. [27], with minor modifications [28].