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**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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## **Abstract**

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'-dichlorodihydrofluorescein-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.

**Keywords:** PCL, Lyophilization, Cryoprotectants, Biocompatibility, Cytotoxicity

## 1. Introduction

During the last few decades, extensive work has been done to develop biodegradable polymers suitable for drug delivery studies and tissue engineering.<sup>1, 2</sup> 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( $\epsilon$ -caprolactone) (PCL), to ensure a specific drug targeting, by both the oral route and the parenteral route.<sup>3-6</sup> 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.<sup>7-9</sup> 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.<sup>9</sup> 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.<sup>10</sup> Besides the nontoxic properties and biodegradability, sufficient chemical and physical stability are crucial requirements for their industrial development.<sup>11, 12</sup> 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.<sup>13, 14</sup> 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.<sup>15</sup> 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.<sup>15-17</sup> To protect nanoparticles from stresses and subsequent agglomeration, a cryoprotectant or lyoprotectant is generally used.<sup>16, 17</sup> 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.<sup>15</sup> 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( $\epsilon$ -caprolactone) micro- and nanospheres.

To date, the literature has described the synthesis of PCL materials using an electrospinning,<sup>18, 19</sup> dip-coating,<sup>20</sup> ring-opening copolymerization,<sup>21, 22</sup> solvent casting and melt blending methods.<sup>23</sup> 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,<sup>24</sup> as well as the obtaining of PCL-based films co-loaded with 5-fluorouracil.<sup>20</sup> 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.

## **2. Materials and Methods**

### 2.1. Materials

Poly( $\epsilon$ -caprolactone) used in experiments was obtained from Lactel Absorbable Polymers (Birmingham, Alabama, USA) 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, USA): 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).

#### *2.1.1. 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  $\mu$ g/ml streptomycin at 37°C in humidified atmosphere and 5 % CO<sub>2</sub>.

### 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. 300 mg commercial granules of poly( $\epsilon$ -caprolactone) have been dissolved in 5 mL of organic solvent (chloroform). After approximately 1h, 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-solvent, PCL precipitates and the solution becomes whitish.

### *2.2.1. 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.

5 mL 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.

### *2.2.2. Freeze drying*

After the freezing, method of lyophilisation was utilized at  $-55^{\circ}\text{C}$  and the pressure of 0.3 mbar. Main drying was performed for 8.5 hours, and final drying for 30 minutes, using Freeze Dryer Christ Alpha 1-2/ LD plus.

### 2.3. 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 saccharose), in terms of disrupting or creating new chemical bonds in the polymer structure. FTIR spectra of the samples were recorded in the range of  $400\text{--}4000\text{ cm}^{-1}$  using a Carl Zeiss SPECORD 75 Spectrometer at  $4\text{ cm}^{-1}$  resolution, using KBr pellet method.

### 2.4. Scanning electron microscopy (SEM)

Morphology imaging was realized using scanning electron microscope 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.

### 2.5. Particle size distribution (DLS)

The size distribution of PCL particles was determined by PSA Mastersizer2000 (Malvern Instruments Ltd., UK). 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.

### 2.6. MTT Assay

The cytotoxicity study was realized using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), according to Mossman et al.<sup>25</sup>, with minor modifications.<sup>26</sup> 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, USA) at a density of 40 000 cells/ml and incubated for 20 hours 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 hours, 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, USA). 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.

### 2.7. 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.<sup>27</sup>, with minor modifications.<sup>28</sup> DCFH-DA readily diffuses through the cell membrane and is hydrolyzed by intracellular esterases to non-fluorescent 2',7'-dichlorofluorescein. In the presence of intracellular ROS it is then rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein. The DCF fluorescence intensity is proportional to the amount of reactive oxygen species formed intracellularly.

HepG2 cells were seeded at a density of 75 000 cells/ml into 96-well, black, tissue culture treated microtiter plates (Nunc, Naperville IL, USA) in five replicates. The cells were incubated for 20 h at 37°C in 5 % CO<sub>2</sub> to attach, then 20 µM DCFH-DA was added for 30 min, the excess of DCFH-DA was then removed, and cells were treated with 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) in PBS. Negative control (non-treated cells), vehicle control (1% emulsion) and positive control (0.5 mM t-BOOH) were included in each experiment. For the kinetic analyses the plates were maintained at 37°C and the fluorescence intensity was determined every 30 min during the 5 h incubation using a microplate reading spectrofluorimeter (Tecan, Genios, Austria) at the excitation wavelength of 485 nm and the emission wavelength of 530 nm.

Statistical significance between treated groups and controls was determined by two tailed Student's t-test and by Kruskal-Wallis one-way analysis of variance (ANOVA) with Dunett's posttest; P<0.05 was considered as statistically significant. Three independent experiments with five replicates were performed.

### **3. Results and discussion**

#### 3.1. Results

##### *3.1.1. Fourier transform infrared spectroscopy*

The infrared spectra of the PCL polymer (Figure 1) show bands characteristic for ester carbonyl (C = O) stretching at 1730 cm<sup>-1</sup>. High intensity peaks at 1160 cm<sup>-1</sup>, 1180 cm<sup>-1</sup> and 1235 cm<sup>-1</sup> represent carboxyl group vibrations (O = C - O). Higher intensity of mentioned bands, compared to others in

spectra, can be explained by the fact that those groups are more polar than others. In the part of spectra with higher wavenumbers, doublet can be observed at  $2910\text{ cm}^{-1}$  and  $2925\text{ cm}^{-1}$ , representing coupled  $\text{sp}^3$  hybridized (C - H) symmetrical and asymmetrical bands. Asymmetrical  $\text{CH}_2$  stretching band is on the spectra at  $2855\text{ cm}^{-1}$ . Peaks on  $1100\text{ cm}^{-1}$ ,  $1040\text{ cm}^{-1}$  and  $955\text{ cm}^{-1}$  are due to (C - O) bond vibrations, while other peaks below  $900\text{ cm}^{-1}$  are from deformational (C - H) vibrations.

Infrared spectra are often sensitive on conformation change and ordering of molecular chains in semi-crystalline polymers, and this ability enables them to be used for crystallinity characterization. (C - O) and (C - C) bonds, characteristic for crystalline state, absorb at  $1360\text{ cm}^{-1}$  and  $1290\text{ cm}^{-1}$ , while band at  $1160\text{ cm}^{-1}$  represents amorphous state of the polymer. Those bands are expected since PCL is a semi-crystalline polymer.  $\text{CH}_2$  deformational bands showed peaks at  $1460\text{ cm}^{-1}$ ,  $1410\text{ cm}^{-1}$  and  $1390\text{ cm}^{-1}$ . Wide band at  $3425\text{ cm}^{-1}$  is a result of combination of (C = O) overtone and hydrogen bonded OH vibrations. Latter bond is very helpful in determining the residual water, as well as the presence of hydrogen bonds. On Figure 1a, of PCL lyophilized without cryoprotection, it can be observed that this bond is of very low intensity. That is also the case for PCL lyophilized with 1% PGA solution (Figure 1c). Other samples (Figure 1b, 1d and 1e) induced higher intensity of OH vibration bands. On the spectra of PCL lyophilized with 1% saccharose solution (Figure 1b), bands are shifted to lower wavenumbers for approximately  $10\text{ cm}^{-1}$ , compared to the spectra of PCL lyophilized without the cryoprotectant. Also, band and intensity changes below  $1200\text{ cm}^{-1}$  clearly indicate to residual saccharose in the sample. When PCL is lyophilized with 1% PGA solution, FTIR spectrum shows the presence of both polymers with carbonyl bond peaks at  $1730\text{ cm}^{-1}$  and  $1710\text{ cm}^{-1}$  (Figure 1c). Bands below  $1200\text{ cm}^{-1}$  are different then the ones in Figure 1a and are characteristic for PGA. The same can be said for the spectra on Figures 1d and 1e, where lower wavenumber bands represent vinyl groups of residual PVA and deformational (C - C - O + C - C - H) groups of glucose, respectively.

### 3.1.2. Scanning electron microscopy

Figure 2 shows SEM images of PCL lyophilized without and with different protective excipients. If no protection is present, it can be seen that after lyophilisation (Figure 2A), particles became massively agglomerated and deformed. Once those images were made, necessity for using cryoprotectants to maintain the integrity of particles was clear. Particles have irregular shapes, and sporadic spheres are present. With the addition of 1% saccharose solution as cryoprotectant into PCL formulation, quite agglomerated cuboid particles have been formed (Figure 2B). In the case of samples obtained with the addition of 1% PGA solution (Figure 2C), spheroidal particles with less agglomeration, compared to previous samples, were obtained. Apart from spheroids, there are also irregular shapes present. Figure 2D shows SEM image of PCL lyophilized with 1% PVA solution. A continuous film of PVA can be observed. This film prevents the observation of individual PCL particles that reside within it. This polymer is one of the most frequently used stabilizers to produce stable nanoparticles. When it comes to PCL polymer, at this concentration, it is not very helpful. If 1% glucose solution is added to the system, SEM images show an obvious uniformity improvement and quite smaller spherical particles compared to all other samples. Particles are less than one micron, smooth surfaced and non-agglomerated (Figure 2E).

In order to confirm i.e. to determine the influence of the lyophilization conditions on morphology of PCL particles prepared with 1% glucose solution, new sample was synthesized. Instead of freeze drying, sample was dried at room temperature and atmospheric pressure for 72 hours. The SEM images obtained for this sample are shown in Figure 3. Assuming that all other parameters of particle preparation were strictly controlled, it is obvious that drying temperature and pressure had substantial effects on particles morphology. Without liophilization, PCL particles, prepared with 1% glucose solution and by drying at room temperature, were micron-sized. From the SEM images (Figure 3) it can be seen that particles have both spherical and irregular shapes and are as well significantly agglomerated.

### *3.1.3. Particle size distribution*

Due to size and uniformity improvements with the addition of 1% glucose solution, size distribution profile was determined for this particular sample (Figure 4). Dynamic Light Scattering method was used to generate size distribution profile (Figure 4B). Results show that 10% of the number of particles is with 362 nm diameter, 50% of particles have diameter less than 520 nm, while 90% of particles are with diameter below 961 nm. Fitting parameter of 1.074% shows good correspondence of calculated with experimental data.

#### *3.1.4. MTT Assay*

Cytotoxicity of the samples was determined by MTT assay, testing 0, 0.0001, 0.001, 0.01, 0.1 and 1 % (v/v) concentrated samples of PCL without cryoprotectants, PCL with 1% PGA or PCL with 1% glucose, exposing HepG2 cells with them for 24 h (Figure 5). Samples PCL without cryoprotectants and PCL with 1% PGA did not significantly reduce the viability of HepG2 cells (Figure 5), while PCL with 1% glucose caused slight decrease in cell viability at 1 % (Figure 5).

#### *3.1.5. DCFH-DA Assay*

The HepG2 cells were pre-treated with DCFH-DA (20  $\mu$ M) for 30 min, washed and then exposed to different concentrations of samples (PCL without cryoprotectants, PCL with 1% PGA, and PCL with 1% glucose) or 0.5 mM t-BOOH as the positive control (PC). DCF fluorescence intensity was measured at 30 min intervals during the 5 h incubation. Sample of PCL without cryoprotectants at concentrations 0.1 and 1 % caused significant increase in DCF fluorescence intensity, which was after 5 hour exposure more than two fold higher from that in control cells (Figure 6), while sample of PCL with 1% PGA solution did not significantly alter DCF fluorescence intensity (Figure 6). Sample of PCL with 1% glucose solution induced timely dependent increase in ROS levels for concentration of 1% (v/v).

### 3.2. Discussion

In the studies of the effect of spherical carriers' size on their circulation half-life, it is often showed that when spherical structures reside within the range of 20 nm - 1  $\mu$ m, all of the clearance

mechanisms are minimized and circulation times are enhanced.<sup>29</sup> Most current nanoparticulate systems are spherical in shape, and extensive work has been dedicated to studying their biological behaviour *in vitro* and *in vivo*. Similar to size, shape is a fundamental property of micro- and nanoparticles that is critically important for their intended biological functions.

Physicochemical solvent/non-solvent method is the starting point of this study. This method is based on the solvent (chloroform) diffusion into non-solvent (ethanol). Presence of ethanol results in a quicker precipitation and phase separation of the PCL polymer, during the diffusion of chloroform. After the solvent evaporation and partial freezing in the freezer, process of freeze drying has been utilised. During the lyophilisation, only three parameters can be controlled: shelves and condenser temperatures, chamber pressure and time. The most important parameter, product temperature, can not be directly controlled. During freezing step, system temperature was maintained at -55°C, which is above the glass transition temperature of PCL, but below the one of cryoprotectants.

Freeze drying process generates various structural modifications of the material. PCL particles freeze dried with no cryoprotection showed erratic morphology with obvious agglomeration (Figure 2A), due to instability of suspended particles and the solvent evaporation. With the addition of PVA, PGA, glucose or saccharose, different processes occur within the formulation.

### *3.2.1. Evaluation of saccharose, PGA, PVA and glucose as different cryoprotectants*

The order of crystallinity depends on the rate of cooling, but also on the polymer chain configuration. Nucleation starts at small, nanometer sized places with preferential organization of polymer chains or their segments. Apart from thermal mechanisms, the addition of impurities can also cause nucleation, which is then termed heterogeneous nucleation. In the case of lyophilized PCL with the addition of 1% saccharose solution, saccharose may have had an effect on the crystallisation of PCL (Figure 2B). Saccharose does not precipitate as a crystal phase, but it becomes supersaturated when cooled below eutectic point.<sup>30</sup> This means that saccharose is highly viscous in the system, so it may have induced nucleation of PCL polymer by steric effects of its double ring. From a diluted solution, crystals can grow in lamellar, layered structures up to 10 µm long. Those structures are often observed as

multilayer structures.<sup>31</sup> Following crystallisation, ordered structures grow so that layers are stacked in lateral dimension. FTIR analysis (Figure 1b) shows increase in intensity of OH vibration bands, suggesting the presence of residual water and hydrogen bonding.

Poly(glutamic acid) is an anionic, hydrophobic, non-toxic and biocompatible biopolymer with glutamic acid in a repeating unit. Under certain circumstances, carboxylic groups of PGA are deprotonated, making it polyelectrolyte. SEM micrographs (Figure 2C) of PCL lyophilized with 1% PGA solution show spheroid particles with sizes above one micron and with irregular shapes. Cryoprotection by PGA can be explained with electrostatic repulsion of double electric layers. Potential energy of particle-particle interaction is a sum of repulsion potential that arises from electric double layer interactions and attraction potential from Van der Waals forces. FTIR spectra of PCL polymer lyophilized with 1% PGA solution shows, apart from residual PGA, that hydrogen bonds are relatively absent from the system.

In the presence of water, PVA generates film.<sup>32, 33</sup> From SEM micrographs, a continuous film can be observed (Figure 2D). This thin PVA film is not allowing the observation of individual particles that reside within it. During nanoparticle preparation, interpenetration of PVA and PCL molecules occurs. Hydrophobic vinyl segments of partially hydrolyzed PVA penetrate into PCL molecules, once molecules of ethanol diffuse. In addition to this, there is a high intensity peak at  $3440\text{ cm}^{-1}$ , in FTIR spectra of PCL lyophilized with 1% PVA solution. Relative sharpness of this peak suggests the presence of intra- and intermolecular hydrogen bonding networks.

Considering the ability to preserve the integrity of PCL particles from the aspect of lyophilisation, the best results are obtained using 1% solution of glucose. Good cryoprotective results could be explained by three widely suggested hypotheses.<sup>34 - 36</sup> Introducing it to the solution, glucose generates hydrogen bond networks between its hydroxyl groups and the carbonyl groups of the PCL polymer segments on the particle's surface. Also, water takes place in this type of bonding. With progressive lowering of the temperature, phase separation emerges. PCL particles, surrounded with glucose molecules, are being separated into a highly viscous unfrozen fraction, pulling a certain amount of water molecules in it. This enables better mechanical protection of PCL particles. Reaching T<sub>g</sub>, glucose experiences a glass transformation, isolating PCL particles within its glassy matrix. Unfrozen

water acts as a plasticiser, reducing crystal pressure and preventing subsequent agglomeration. In addition, multi-hydroxyl components are able to maintain a spatial orientation and distance between individual particles after the sublimation of ice. FTIR spectra show a very high level of hydrogen bonding and the presence of OH bonds.

### 3.2.2. *In vitro* studies

The aim of this study was also to inspect the potential toxicity of PCL particles, lyophilised with different cryoprotectants or without any. Even though *in vitro* studies are not a complete replacement for animal models, using simple models provides insight to general toxicity mechanisms and can be a basis for further analysis.

Mitochondrial redox activity of HepG2 liver cells to reduce MTT to formazan was investigated, after treating with different samples of PCL polymer. In this test, only live cells can generate a strong signal, while dead cells are inactive and unable to produce any signal. Samples of PCL without cryoprotectant and PCL with 1% PGA did not significantly reduce the viability of HepG2 cells (Figure 5), while PCL with 1% glucose caused minor damage to cells at 1 % sample concentration (Figure 5).

Investigating toxicity profile, it is not always clear how particles behave in the exposed environment. Do cells internalize particles through endocytosis and by which mechanisms, are just few of many problems that need to be addressed. Apart from size, shape also has a crucial effect on cell adhesion and overall interaction. It has been shown that particles with different shapes undergo different types of endocytosis. The same case is with particles of different sizes, showing that spherical particles of 200 nm diameter are internalized by clathrin mediated endocytosis, while particles of 500 nm and above, undergo caveolar endocytic pathways.<sup>37</sup> Therefore, PCL particles lyophilized with 1% glucose solution which have more spherical shapes, smaller sizes, improved uniformity and smoother surfaces compared to ones lyophilized with 1% PGA solution, contributed to better internalization. Knowing that internalized particles have more chances of disrupting the

functionality of cells, the explanation of cell viability reduction after exposure of HepG2 cells to 1% v/v of PCL particles obtained with 1% glucose is obvious.

PCL samples without cryoprotectant at 0.1 and 1 % concentration caused significant increase in DCF fluorescence intensity, which was after 5 hour exposure more than two fold higher from that in control cells, while sample of PCL with 1% PGA did not significantly alter DCF fluorescence intensity (Figure 6). At first glance, time dependent increase in ROS levels are observed in cells by PCL particles lyophilized with 1% glucose solution. Negative control also produced a certain amount of ROS, suggesting that there was something else in the experiment that gave rise to ROS levels. Figure 6 shows that there was no significant increase in DCF fluorescence intensity, apart from 1% concentrated sample. The effects of the highest concentrated sample can be explained with increased internalization and subsequent harmful interaction of particles with organelles inside the cells.

Most cells produce a certain amount of ROS in metabolic processes or as a defence mechanism. When that amount is significant, cells are prone to various negative effects such as oxidative stress, leading to many diseases. However, link between ROS levels and the induction of toxic effects depends on the cell type and is not yet well investigated.

#### **4. Conclusion**

Nanometre scaled spherical PCL particles, with narrow size distribution and with smooth surfaces were successfully synthesized by physicochemical solvent/non-solvent method and freeze drying process. The influence of different cryoprotective excipients (glucose, saccharose, polyvinyl alcohol or polyglutamic acid) during freeze drying process was evaluated. Solution of 1% saccharose induced crystallisation, while 1% PVA solution formed film, suggesting that at 1% concentration, those substances are not always helpful in the preparation of redispersable PCL nanoparticles. When 1% PGA solution was added to the formulation, freeze drying produced microspheres with irregularities. The best results were achieved using 1% solution of glucose. From size distribution profile, it can be seen that 90% of the PCL particles obtained with 1% solution of glucose are less than 960 nm in diameter. Biological assays showed improved internalization by liver HepG2 cell line. It can be



concluded that physicochemical solvent/non-solvent method, with freeze drying process and 1% glucose solution, is a good way of obtaining uniform, but also biocompatible PCL nanospheres for biomedical purposes.

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## Figure captions

**Figure 1.** FTIR spectra obtained from: **a)** PCL lyophilized without cryoprotectant; **b)** PCL lyophilized with 1% saccharose solution; **c)** PCL lyophilized with 1% PGA solution; **d)** PCL lyophilized with 1% PVA solution and **e)** PCL lyophilized with 1% glucose solution.

**Figure 2.** Representative SEM images of the samples produced with different cryoprotectants **A)** PCL without any cryoprotectant, **B)** PCL lyophilized with the addition of 1% saccharose solution, **C)** PCL with 1% PGA solution, **D)** PCL with 1% PVA solution and **E)** PCL lyophilized with the addition of 1% glucose solution.

**Figure 3.** Representative SEM images of PCL particles dried at room temperature and prepared with the addition of 1% glucose solution. The micrographs are given for comparison purposes.

**Figure 4.** **A)** SEM Image: PCL lyophilized with the addition of 1% glucose solution, with marked approximate particle sizes and **B)** DLS size distribution profile: PCL particles lyophilized with 1% glucose solution.

**Figure 5.** Viability of HepG2 cells treated with PCL without cryoprotectants (**blue**), PCL with 1% PGA (**green**) and PCL with 1% glucose (**red**) for 24 h. The data are presented as mean values of three independent experiments (each with five replicates)  $\pm$  S.D. (\*) denotes a significant difference between samples-treated groups and control (Student's t-test,  $P < 0.05$ ).

**Figure 6.** PCL without any cryoprotectant (a), PCL with 1% PGA solution (b) and PCL with 1% glucose solution (c) - induced intracellular ROS formation in HepG2 cells. The HepG2 cells were pre-treated with DCFH-DA (20  $\mu$ M) for 30 min, washed and then exposed to different concentrations of PCL (0, 0.001, 0.01, 0.1 and 1 %) or 0.5 mM t-BOOH as the positive control (PC). DCF fluorescence intensity was measured at 30 min intervals during the 5 h incubation. (Top) kinetic of ROS formation during 5 hour exposure; each point represents the mean of five replicates ( $\pm$ SD) of representative experiment; (\*) denotes a significant difference between sample-treated groups and control (Student's t-test,  $P < 0.05$ ), (Bottom) fold increase of ROS level in exposed cells over the level in control cells after 5 hour exposure; each bar represent means ( $\pm$ SD) of three independent experiments. (\*) denotes a significant difference between sample-treated groups and control (Kruskal - Wallis one-way analysis of variance (ANOVA) with Dunett's post test).

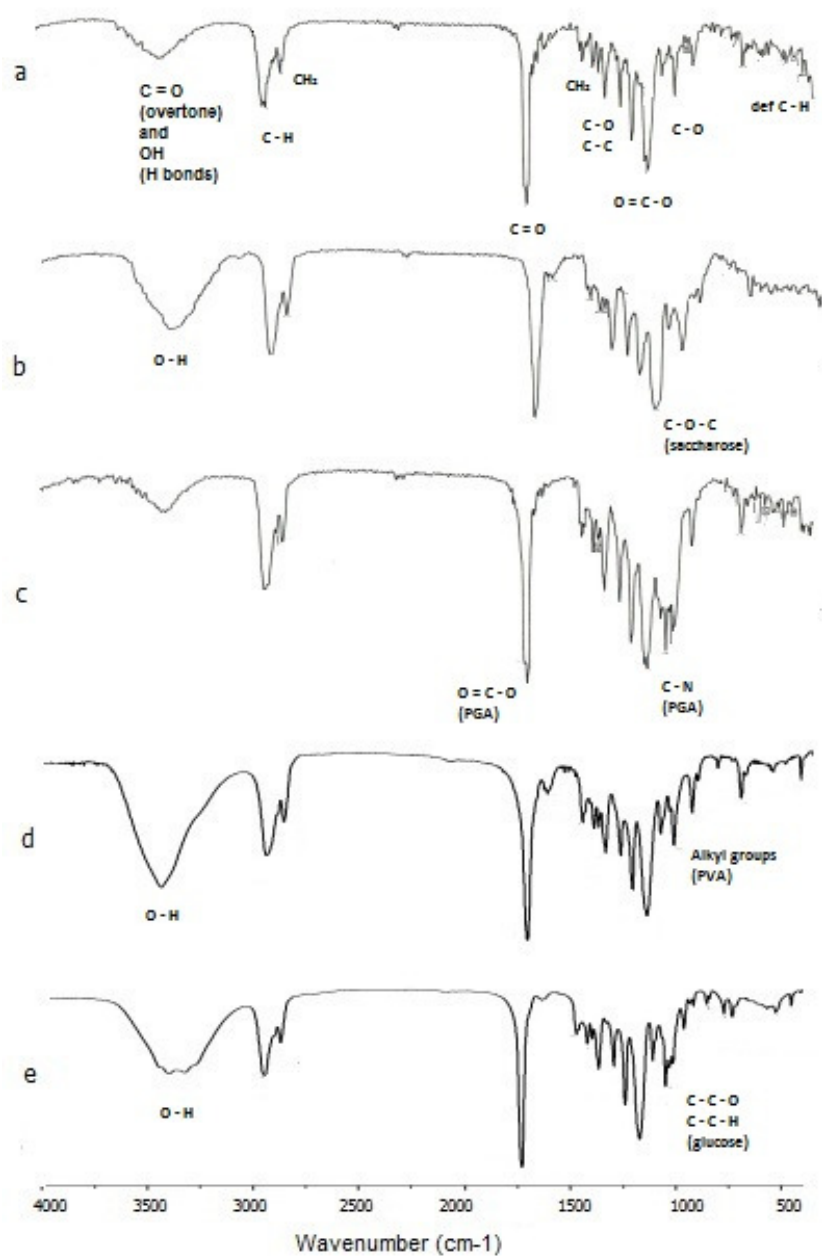
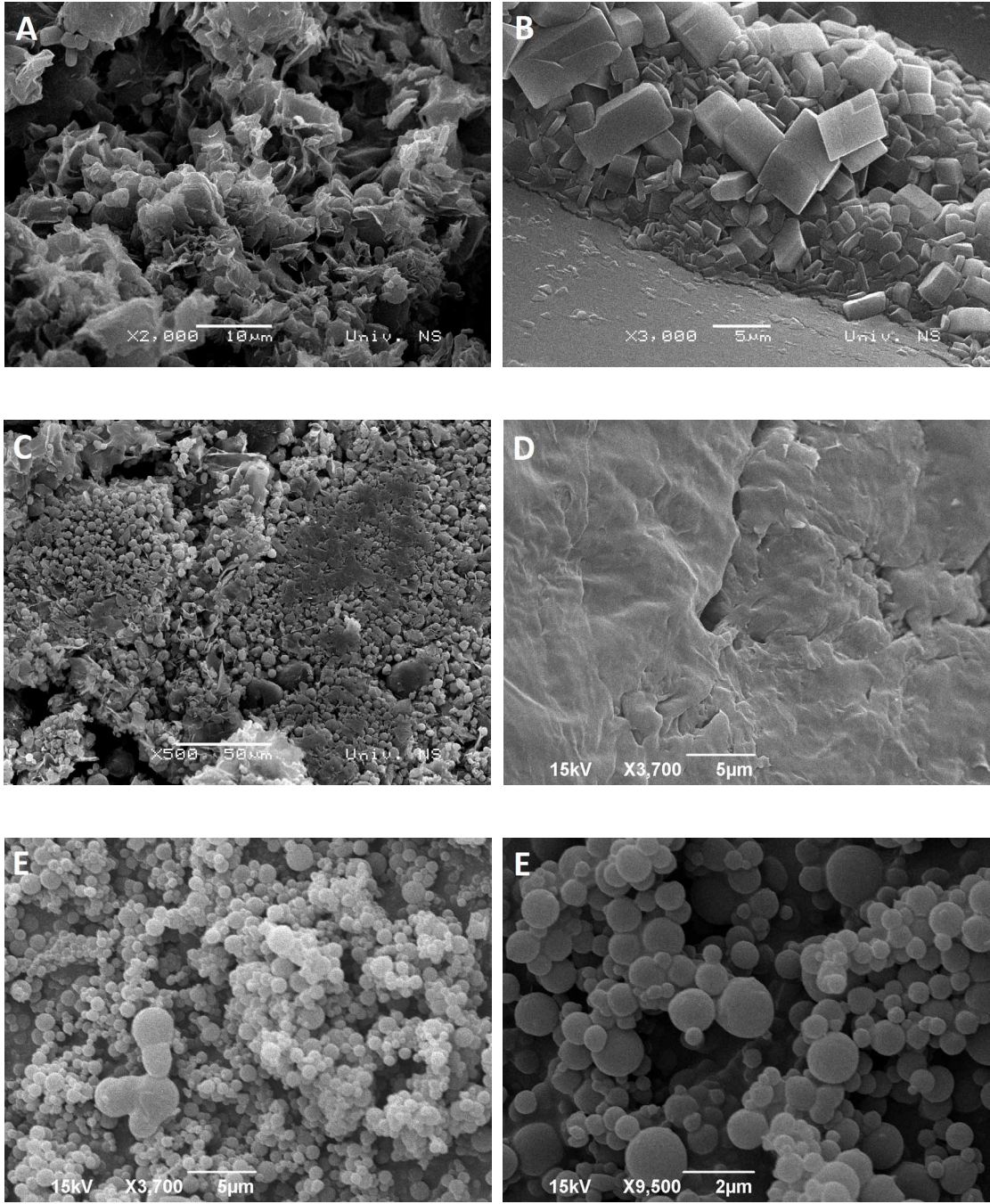
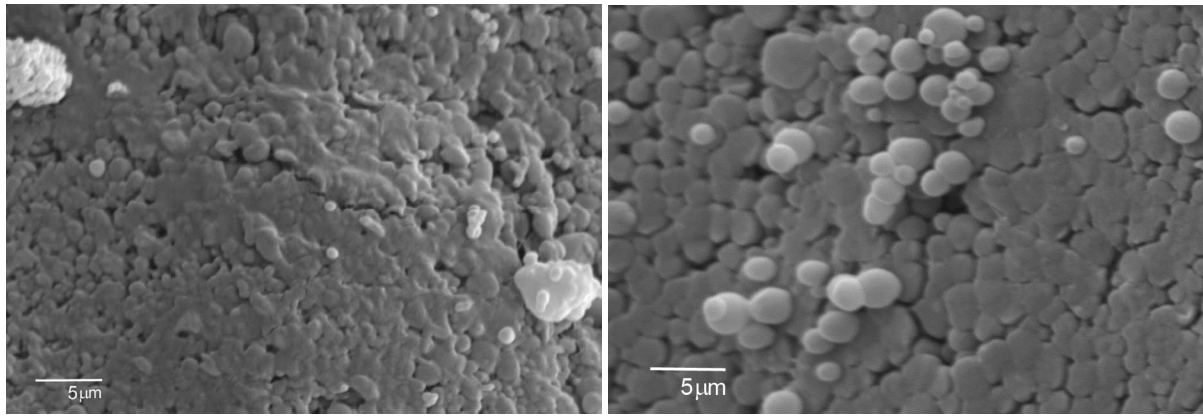


Figure 1.

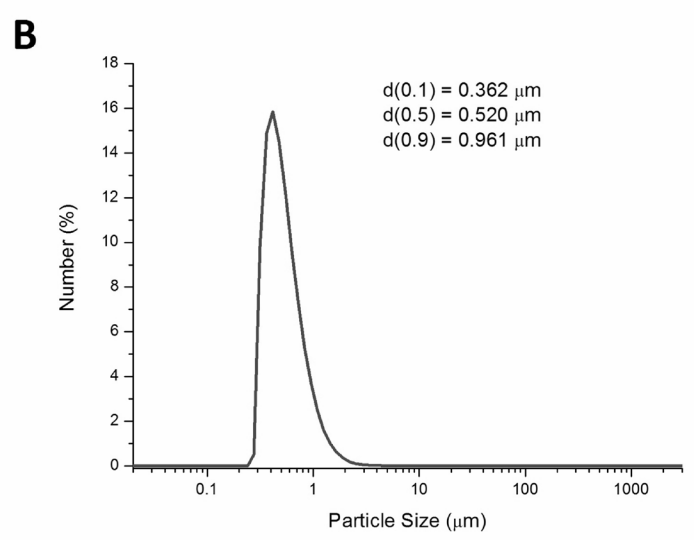
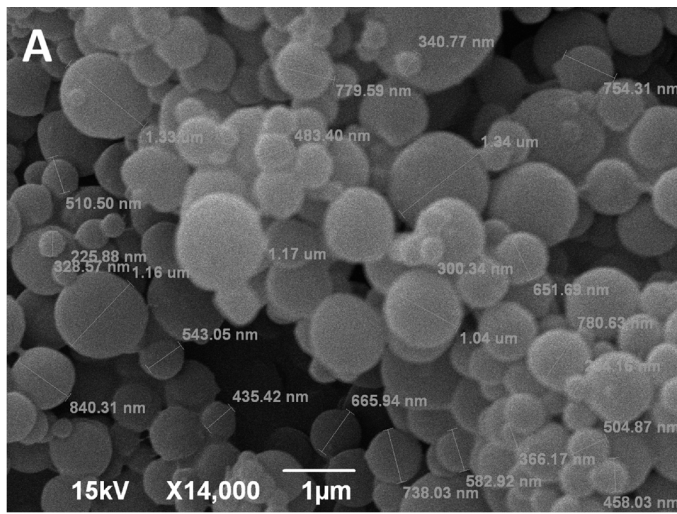


**Figure 2.**



**Figure 3.**





**Figure 4.**

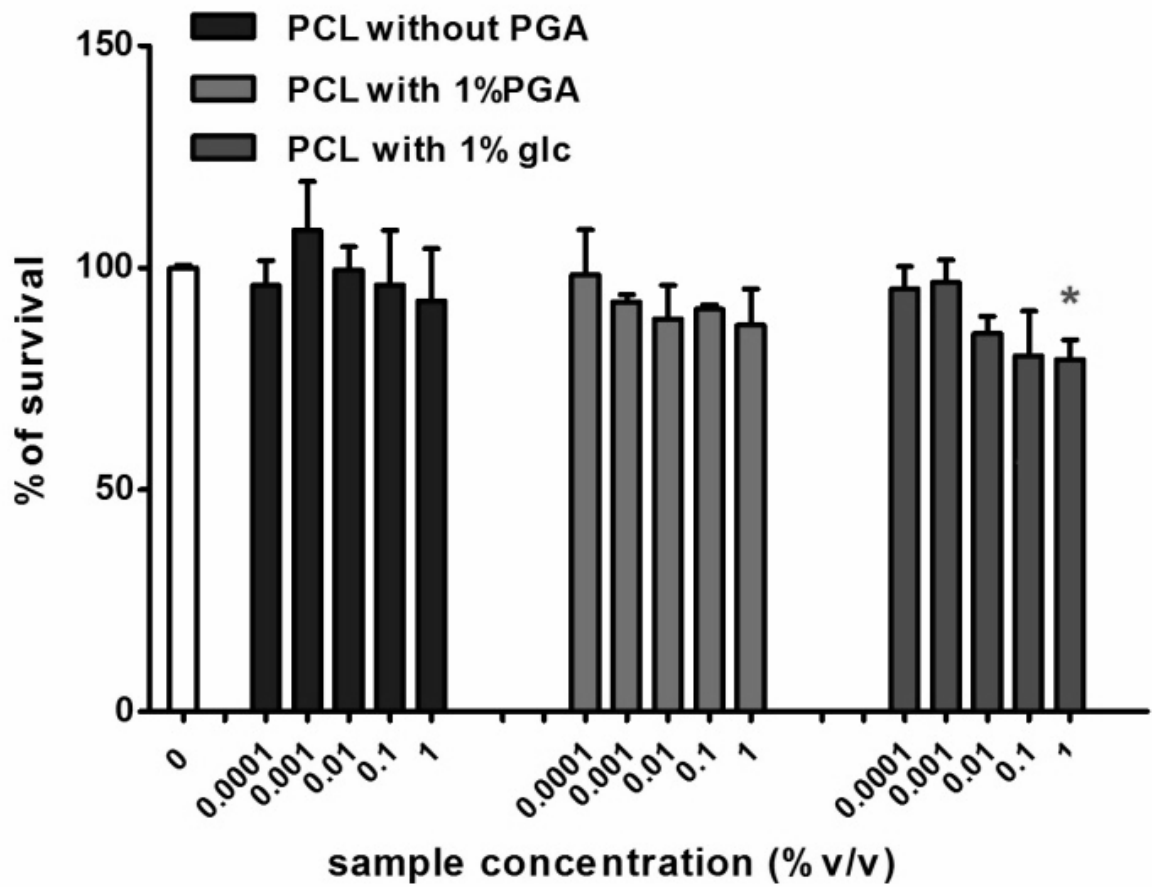


Figure 5.

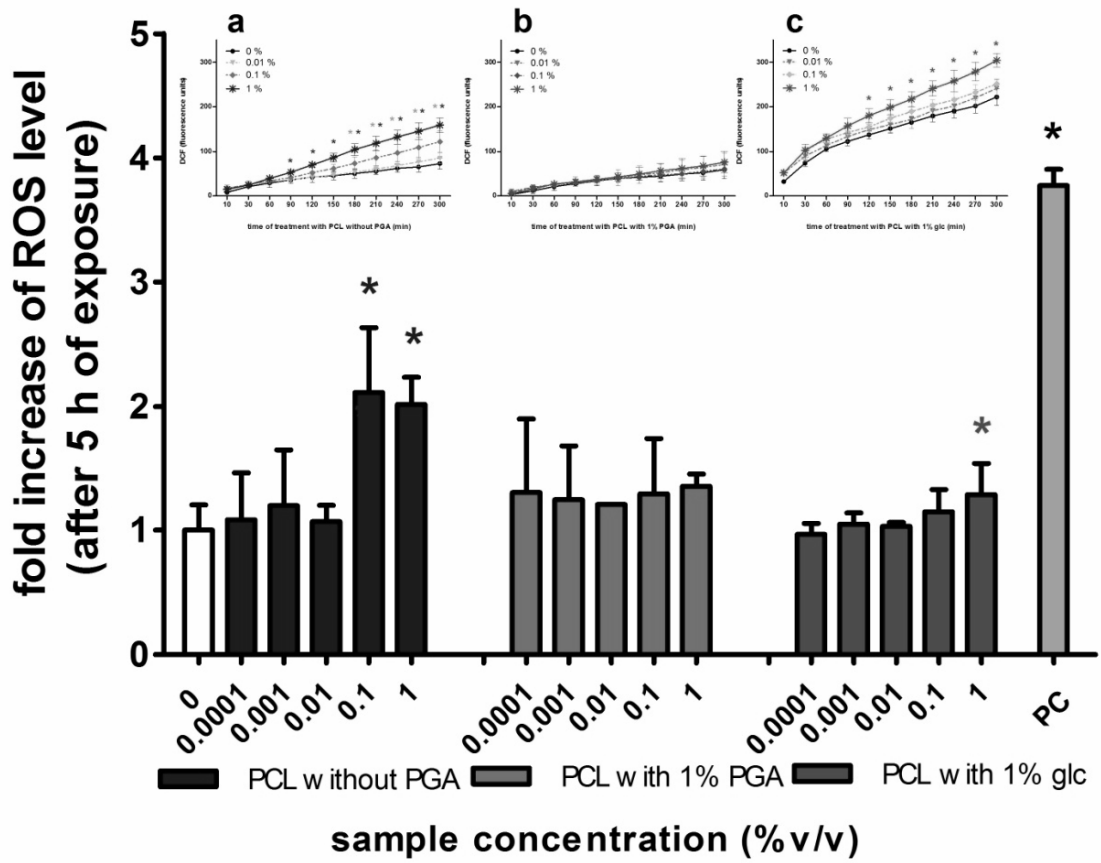


Figure 6.