

## **Effects of sterilization techniques on the PEGylated poly ( $\gamma$ -benzyl-L-glutamate) (PBLG) nanoparticles**

### **Sterilizasyon tekniklerinin PEGlenmiş poly ( $\gamma$ -benzil-L-glutamat) (PBLG) nanopartiküller üzerine etkileri**

**İpek Özcan<sup>1\*</sup>, Kawthar Bouchemal<sup>2</sup>, Freimar Segura-Sánchez<sup>3</sup>, Özlem Abacı<sup>4</sup>,  
Özgen Özer<sup>1</sup>, Tamer Güneri<sup>1</sup>, Gilles Ponchel<sup>2</sup>**

<sup>1</sup>Ege University, Faculty of Pharmacy, Department of Pharmaceutical Technology, 35100 Izmir, Turkey.

<sup>2</sup>Paris-South University, UMR CNRS 8612, Faculty of Pharmacy, Physicochimie Pharmacotechnie Biopharmacie, 92290 Chatenay-Malabry, France.

<sup>3</sup>Universidad de Antioquia, Facultad de Química Farmacéutica, Departamento de Farmacia, Medellín, Colombia.

<sup>4</sup>Ege University, Faculty of Science, Department of Biology, Basic & Industrial Microbiology, 35100 Izmir, Turkey.

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#### **Abstract**

Nanoparticles were prepared by nanoprecipitation technique using poly ( $\gamma$ -benzyl-L-glutamate) (PBLG) derivatives. Injectable nanoparticles were assessed for their in vitro properties such as particle size, polydispersity index, zeta potential and surface properties. The possibilities of sterilizing PBLG nanoparticle suspensions by membrane filtration or autoclaving were evaluated. It was found that among these techniques, sterilization with membrane filtration seemed to be the most appropriate technique with any significant effect on in vitro and surface properties. Sterility was assessed on the final product according to pharmacopoeial requirements. Sterility testing results showed no microbial contamination indicating that sterile nanoparticle formulations have been achieved after membrane filtration.

**Key words:** Poly ( $\gamma$ -benzyl-L-glutamate), PEGylated nanoparticles, membrane filtration, autoclaving, sterility testing.

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#### **Introduction**

Application of nanoparticles as drug carriers offers several advantages such as targeting, stealth properties, efficient drug loading and controlled drug release (Mohanraj and Chen 2006, Park 2007). Ideally, such particles should be based on a family of polymers constituted of the same backbone and bearing adapted chemical groups. The polymers should then be combined on demand for conferring the desired properties to the nanoparticles (Soppimath et al. 2001). In particular, poly ( $\gamma$ -benzyl-L-glutamate) (PBLG) nanoparticles appear to be a promising and versatile drug delivery system. Because various chemical moieties could be quite easily introduced in the PBLG structure to form various copolymers (Barbosa et al. 2008, Tsukada et al. 2009). PEGylated nanoparticles exhibited a long circulation time in blood due to reduced opsonization.

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\*Corresponding author: ipek.ozcan@ege.edu.tr

For this purpose, many studies have been reported about polymer and hydrophilic PEG grafting (Peracchia et al. 1998, Veronese 2001, Jeong et al. 2005, Kim et al. 2007).

Sterilization of nanoparticles is a major challenge in the designing of appropriate drug delivery systems. For clinical use, parenteral drug delivery systems have to meet the pharmacopoeial requirements of sterility. The chemical or physical lability of the polymer matrix usually limits most conventional methods for obtaining acceptable sterile products (Athanasidou et al. 1996, Alleman et al. 1998). A satisfactory sterilization technique, able to keep intact the supramolecular and molecular structure of the colloids, is a major challenge in the case of polymer nanoparticles used as drug carriers.

With chemical sterilization by gases such as ethylene oxide, toxicological problems may be encountered due to toxic residues. Numerous studies have shown the effects of  $\gamma$ -irradiation on the stability and the safety of colloidal carriers based on polyesters, principally microparticles and nanoparticles (Volland et al. 1994, Mohr et al. 1999, Memisoglu and Hincal 2006). Therefore, the selection of a suitable sterilization method for such type of formulations is crucial to ensure their physical and chemical integrity, their performance and safety in vivo.

As an alternative technique, sterile filtration based on physical removal of contained microorganisms through 0.22  $\mu\text{m}$  membrane filters may be considered as the appropriate method for chemically or thermally sensitive materials since it has no adverse effect on the polymer and the drug (Goldbach et al. 1995, Konan et al. 2003, Maksimenko et al. 2008). Moreover, it has the advantage of not adversely affecting the drug release properties and the stability of a formulation nor the chemical stability of ingredients (Chorny et al. 2004). Nevertheless, this sterilization method can only be used for nanoparticles with a mean size significantly below membrane cut-off and with a narrow size distribution to avoid membrane clogging. However, this technique is not suitable for larger nanoparticles (exceeding 200 nm) when the drug is adsorbed at the nanoparticles surface or when the colloidal suspensions are too viscous (Alleman et al. 1993, Zheng and Bosch 1997, Brigger et al. 2003, Tsukada et al. 2009).

Heat sterilization by autoclaving is a highly effective technique involving high temperatures, which may influence decomposition or degradation of active ingredient as well as the microparticle or nanoparticle material, i.e., polymer (Memisoglu and Hincal 2006, Wörle et al. 2006). A significant increase in particle size was reported after autoclaving of the unloaded polybutylcyanoacrylate nanoparticle suspensions and the nanoparticle powders were characterized by impaired resuspension characteristics. These were attributed to the swelling of polymeric membrane (Sommerfeld et al. 1998). Sterilization by autoclaving induces a degradation of polyesters by hydrolysis and these polymers are also heat sensitive due to their thermoplastic nature (Konan et al. 2002). On the other hand, solid lipid nanoparticles (SLN) can be sterilized by autoclaving, maintaining an almost spherical shape, without any significant increase in size or nanoparticle distribution (Schwarz et al. 1994, Cavalli et al. 1997, Sanna et al. 2003). The Food and Drug Administration requires that sterile pharmaceutical products be free of viable microorganisms. Sterility testing of pharmaceutical products provides added assurance that the product is sterile. Sterility testing is typically done by inoculating the drug product into microbial growth media including Gram-positive and Gram-negative bacteria,

sporeforming bacteria, yeast and fungi followed by visual inspection for growth during incubation for a specified time period. A lack of visual growth indicates that the drug product samples tested were sterile (Workman and Clayton 1996).

In the present work, the effect of sterilization technique on PBLG nanoparticle properties such as particle size, zeta potential, polydispersity index were assessed. The effectiveness of the sterile filtration on the particle surface properties was also evaluated based on isothermal titration calorimetry measurements. Moreover, microbiological tests were performed, to verify that the sterilized product complies with the pharmacopoeial requirements for sterility.

## **Materials and Methods**

### *Materials*

PBLG derivatives (PBLG-benzylamine, PBLG-Bnz) were synthesized by ring-opening polymerization of  $\gamma$ -benzyl-L-glutamate N-carboxyanhydride (ISOCHEM-SNPE) using selected amine-terminated initiators and analyzed previously (Barbosa et al. 2007, Ozcan et al. 2008). Commercial PBLG (PBLG-com) was purchased from Sigma-Aldrich. Methoxy poly (ethylene glycol) amine (mPEG-NH<sub>2</sub>) was obtained from Shearwater Corporation. Water was purified by reverse osmosis (MilliQ, Millipore). All other reagents were of analytical grade and were used as received.

### *Preparation of nanoparticles*

The surfaces of the nanoparticles were modified with polyethylene glycol (PEG) (Shearwater Corporation) as hydrophilic agent. F1 and F2 coded nanoparticles were prepared by nanoprecipitation technique using composite polymers (PBLG-Bnz/PBLG-PEG and PBLG-com/PBLG-PEG) at a ratio of 2:1, respectively (Thioune et al. 1997). Nanoparticles prepared with only PBLG-Bnz and PBLG-com were used for control experiments as non-PEGylated nanoparticles. Briefly, 15 mg of polymer or polymer mixture was dissolved in 5 mL of tetrahydrofuran at 30 °C. This solution was added by dripping to 10 mL of milli-Q water under magnetic stirring. Then the mixture was transferred in a Teflon surface. The solvent was evaporated, at 30 °C, under a light air flow. Nanoparticles were washed with 5 mL of milli-Q water and evaporation was carried out to yield 10 mL of nanoparticles suspension.

### *Sterilization of nanoparticles*

#### *Membrane filtration*

The formulations were filtered through a sterile 0.22  $\mu$ m Milipore Express™ membrane filter (Millipore®, Volketswil, Switzerland). Afterwards, sterile suspension was poured into sterile glass vials.

#### *Autoclaving (Heat sterilization)*

Samples after preparation were divided into two groups of equal volume. They were put into glass vials, which were sealed with rubber stoppers and aluminum caps. One group was sterilized at 121 °C for 20 min in thermo-controlled autoclave (ALFA Junior) while the other group (reference) was kept at 8 °C for comparative evaluation of physicochemical properties with their sterilized analogue.

### *Physicochemical characterization of nanoparticles*

The mean diameter and particle size distribution was determined before and after sterilization by dynamic laser light scattering (Nanosizer Coulter N4 Plus®) and also their observation in transmission electron microscopy (TEM-Philips EM 208). Zeta potential measurements were carried out using a Zetasizer 4, Malvern Instrument. Each sample was measured in triplicate.

### *Isothermal titration calorimetry (ITC) experiments*

PEGylated and non PEGylated, sterile and non-sterile nanoparticle-protein interactions were measured using bovine serum albumin (BSA) as a model globular protein by ITC (VP-ITC, MicroCal®). BSA solution was placed in continuously stirred syringe at 270 rpm and nanoparticle suspension was placed in the sample cell. Injections of 10  $\mu$ L of the BSA solution were made at intervals of 10 min. Ultrapure

water used as a control experiment. Experiments were carried out at 25 °C. These results allowed us to demonstrate differences in the surface properties of PEGylated and non-PEGylated nanoparticles in suspension after membrane filtration (Bouchemal 2008).

#### *Microbial sterility testing of formulations*

The sterility testing was performed on the nanoparticles, following USP XXIII guidelines, on the nanoparticle suspensions. Thioglycollate resazurine broth (BioMerieux®, Marcy, France) was used as anaerobic medium for the detection of bacteria and tripcase soy broth was used as medium for the detection of yeasts and fungi. Media were sterilized at 121°C/ 1.1 atm for 15 min. Then, sterile nanoparticles were immersed in tubes containing appropriate media. The tubes were incubated for 14 days at 37°C (thioglycollate resazurine medium) or at 25 °C (tripcase medium). Non-sterile nanoparticles were used as positive controls. The turbidity of the media was then observed over a basic period of 14 days in comparison to positive controls. The experiment was done three times.

#### *Statistical analysis*

The data obtained in this study was subjected to statistical analysis by using analysis of variance (ANOVA) followed by Tukey's HSD multiple comparison test. The SPSS 11.01 statistical software was used. Significance was accepted at a probability level of  $p < 0.05$ .

## **Results and Discussion**

Characterizations of nanoparticles were evaluated on non-sterile and sterile samples. For the sterile PBLG nanoparticles, no variations of the visual appearance were seen after sterile filtration. Table 1 shows the results of measuring the particle sizes (PS), polydispersity index (PI) and zeta potential (ZP) values after different sterilization treatments.

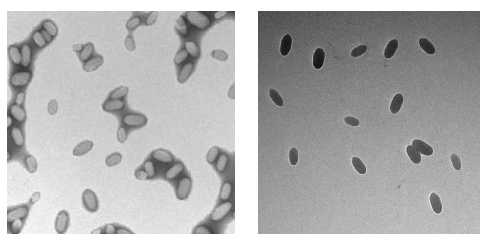
**Table 1.** Physicochemical characteristics of nanoparticles after different sterilization techniques (n=3).

| Formula Code | No sterilization |            |            | After sterilization |            |            |             |            |            |
|--------------|------------------|------------|------------|---------------------|------------|------------|-------------|------------|------------|
|              | PS (nm)          | ZP (mV)    | PI         | Membrane filtration |            |            | Autoclaving |            |            |
|              |                  |            |            | PS (nm)             | ZP (mV)    | PI         | PS (nm)     | ZP (mV)    | PI         |
| F1           | 52±12            | -28.6±0.77 | 0.128±0.03 | 50±10               | -28.4±0.65 | 0.122±0.05 | 120±11      | -20.2±0.55 | 0.425±0.15 |
| F2           | 50±15            | -27.5±0.81 | 0.114±0.05 | 48±16               | -28.5±0.78 | 0.112±0.04 | 115±16      | -22.5±0.32 | 0.342±0.16 |

As seen in Table 1, membrane filtration does not seem to have any significant effect on particle size and polydispersity index ( $p < 0.05$ ). The physical properties for membrane sterilized nanoparticles were close to that of the non-sterilized control sample. Also, slight changes were seen in zeta potential of sterilized nanoparticles ( $p < 0.05$ ). With regard to surface charge, maximal differences of  $-1$  mV comparatively to the non-sterilized reference were measured. The clogging doesn't observed because of the particle size is significantly smaller than the membrane pore size. Masson et al. (1997) have also studied the effect of sterile filtration on the polymeric nanospheres. The poly ( $\epsilon$ -caprolactone) nanospheres which are below 200 nm and have low viscosity could be sterilized by membrane filtration without measurable physical properties loss. However, alternative technique, autoclaving, has a direct impact on nanoparticle size. Autoclaving caused massive aggregation for the nanoparticles followed by precipitation, which led to the conclusion that excessive heat disrupted nanoparticle integrity. Nanoparticles tend to aggregate at high temperature employed in autoclaving which leads to increased particle size and polydispersity index ( $p > 0.05$ ). Rollot et al. (1986) studied with polybutylcyanoacrylate nanoparticles and particle sizes were increased from 200 to 500 nm after autoclaving nanoparticles. Gokce et al. (2008) also reported an increment in particle sizes and a decrement of zeta potential for SLN formulations for the temperatures (110 °C and 121

°C). In other study showed the sterilization of nanoparticles in suspension by autoclaving (121°C, 10 min) produced an irreversible loss of their stability due to aggregation and sedimentation (Sommerfeld et al. 1998).

TEM images also confirmed the dimensions and shape of nanoparticles (Figure 1a, b). In accordance with these results, it could be seen that F1 coded non-sterile nanoparticles exhibited ellipsoidal geometries (Figure 1a). It was clear from TEM image that the shapes of nanoparticles were well conserved after membrane sterilization (Figure 1b).



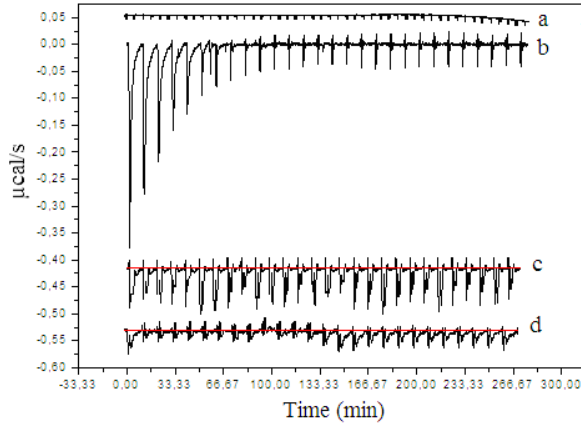
(a) (b)  
**Figure 1.** TEM images of (a) non-sterile nanoparticles; (b) sterile nanoparticles.

After the sterilization of nanoparticles, in order to evaluate capacity of nanoparticles to avoid protein adsorption and to confirm the presence of PEG chains at the surface of PBLG nanoparticles isothermal titration calorimetry has been used. Figure 2 shows the measured sterile/ non-sterile PEGylated and non-PEGylated nanoparticle-protein interactions by ITC.

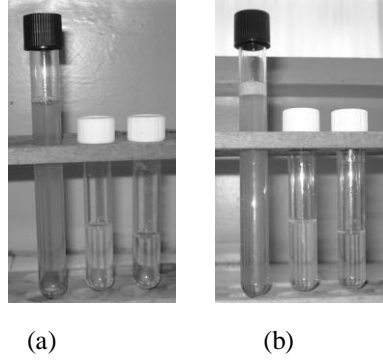
No significant signals were produced by BSA control experiment with ultrapure water (Figure 2a). The results showed that interaction of BSA with the nanoparticle surface is an exothermic phenomenon and the signals registered after each BSA injection were stronger with non-PEGylated nanoparticles than with PEGylated nanoparticles (Figure 2b, c). These results allowed us to demonstrate differences in the surface properties of nanoparticles in suspension. The weak signals were registered with sterile PEGylated nanoparticles (Figure 2d). This was clearly in the case with covalently attached PEG at the surface of nanoparticles, which displayed no surface variation after membrane sterilization.

In order to ensure the sterility of the final nanoparticles (i.e. free of all forms of viable microorganisms), the sterility testing was performed according pharmacopoeial requirements. Sterility was assessed by the observation of the media during the incubation period.

All tested sterile nanoparticles showed no detectable visible growth of microorganisms at the end of 14 days for three different incubation medium (Figure 3a, b). Contrarily to non-sterile formulations as positive controls for which a substantial increase of turbidity was systematically observed.



**Figure 2.** Plots of microcalorimetric titration of nanoparticles (a) control by BSA solution, (b) non-PEGylated nanoparticles, (c) non-sterile PEGylated nanoparticles, (d) sterile PEGylated nanoparticles.



**Figure 3.** Photos of incubation medium with sterile F1 coated nanoparticles (a) 1<sup>st</sup> day; (b) 14<sup>th</sup> day.

## Conclusion

Sterilization is a crucial process for the injectable formulations especially for industrial scaling up. In general, the sterilization of nanoparticle formulations is problematic. It can be concluded that membrane sterilization is the promising technique which did not modify physically or chemically PEGylated PBLG nanoparticles, regardless of their surface characteristics. The sterilization of these nanoparticle formulations by autoclaving is not advisable. These results will be used for the successful development of drug loaded nanoparticulate formulations intended for parenteral administration in future studies.

## Özet

Nanopartiküller, poli ( $\gamma$ -benzil-L-glutamat) (PBLG) türevleri kullanılarak nanoçöktürme yöntemi ile hazırlanmıştır. Enjektabl nanopartiküllerin partikül boyutu, polidisperslik indisi, zeta potansiyel gibi in vitro özellikleri ve yüzey özellikleri tayin edilmiştir. PBLG nanopartikül süspansiyonlarının sterilizasyonları membran filtrasyon ve otoklav kullanılarak değerlendirilmiştir. Bu yöntemlere göre, membran filtrasyon ile yapılan sterilizasyonun nanopartiküllerin in vitro ve yüzey özellikleri üzerine hiçbir anlamlı etkisi olmaması nedeniyle en uygun yöntem olduğu bulunmuştur. Farmakope kurallarına göre final ürün üzerinde sterilite değerlendirilmiştir. Sterilite test sonuçları, membran filtrasyondan sonra

mikrobiyal kontaminasyon göstermeyen steril nanopartikül formülasyonlarının hazırlanabileceği göstermiştir.

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