The Stenlying Effect of High Hydrostatic Pressure on Thermally and Hydrolytically Labile Nanosized Carriers

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Purpose. To investigate whether high hydrostatic pressure (HHP) treatment allows the sterilization of thermosensitive polymer nanoparticle suspensions without jeopardizing their physicochemical integrity.

Methods. Application of HHP was explored on a wide variety of thermosensitive poly(cyanoacrylate) nanoparticles, varying by their type (nanospheres or nanocapsules), by their preparation method (nanoprecipitation or emulsion/solvent evaporation), as well as by their surface characteristics. Physicochemical characterization before and after pressurization included turbidimetry, size measurement, zeta potential, scanning electron microscopy and infrared analysis. A sterility test also conducted according to pharmacopoeial requirements on an importantly contaminated nanoparticle suspension.

Results. Poly(cyanoacrylate) nanoparticles appeared to be extremely baroresistant. Continuous or oscillatory HHP treatment up to 500 MPa during 30 min induced generally neither physical, nor chemical damage. However, precautions should be taken when surface modifiers are adsorbed onto nanoparticles, as a layer destabilization may occur. Finally, this process allowed the successful inactivation of vegetative bacteria, yeast, and fungi.

Conclusions. This work proposes HHP as a new method for polymer drug carriers sterilization, taking into account that further exploration in this area is needed to propose novel protocols for spores inactivation.

KEY WORDS: high hydrostatic pressure processing; nanoparticles; poly(cyanoacrylate); sterilization; surface modifiers.

INTRODUCTION

For clinical use, parenteral drug delivery systems have to meet the pharmacopoeial requirements of sterility. In the case of polymer nanoparticles used as drug carriers, a satisfactory sterilization technique, able to keep intact the supramolecular and molecular structure of the colloids, has not yet been developed.

Polymer nanoparticles may be prone to degradation at elevated temperatures due to the generally low glass-transition temperature of the polymers and surface modifiers they contain (1,2). Hence, subsequent alteration of the physical and mechanical properties of nanoparticles, like aggregation (3), flocculation, acceleration of Ostwald ripening (4), and other mechanisms, make thermal heat sterilization (by steam or dry heat) a difficult approach. Moreover, heat treatment may accelerate hydrolysis reactions, which may induce chemical modifications (2).

Chemical sterilization by gases (formaldehyde or ethylene oxide) also proved to be an unsuitable sterilization process. For instance, no sterilized formulation could be appropriately redispersed for intravenous injection, after formaldehyde treatment at 60°C on freeze-dried poly(butylcyanoacrylate) nanoparticles prepared with different stabilizers (3). In addition, harmful gas residues may remain on the surface or within the nanoparticles, causing them to fail *in vivo* (hemolysis).

Moreover, sterilization by ionizing radiation is also questionable. Although the size of poly(ε-caprolactone) nanoparticles was not altered after this type of treatment, an increase of M_w caused by cross-linking between polymer chains, or even between polycaprolactone chains and surfactants, was reported (5). Poly(lactic acid) (PLA) nanoparticles also displayed an unchanged size after γ-irradiation, but polymeric chain scission was observed. This resulted in an increase in the release rate of the encapsulated drug savopexine (6). In addition, a faster biodegradation and a shorter useful lifetime of PLA nanoparticles should be expected *in vivo* (2).

As an alternative to the previous techniques, physical removal of contained microorganisms by filtration was a soft and feasible process for sub-200 nm nanoparticle suspensions sterilization (7,8). However, this technique is not suitable for larger nanoparticles when the drug is adsorbed at the nanoparticles surface or when the colloidal suspensions are too viscous.

Finally, if it was successfully achieved (9), aseptic preparation of polymeric nanoparticles can be a complex and risky process.

Therefore, there is an urgent need to develop new processes for sterilizing injectable polymer nanoparticles.

Such processes should be applicable to a wide range of nanoparticle suspensions, regardless of their size, their polymeric composition, their morphology (nanospheres or nanocapsules), and the excipients present in their dispersion medium. These processes should be able to reach the critical objective of destroying high levels of microorganisms, includ-

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ABBREVIATIONS: cfu, colony forming unit; HHP, high hydrostatic pressure; MSC, multiplicative scatter correction; PCA, principal component analysis; PHDCA, poly(hexadecylcyanoacrylate); PEG, poly(ethylene glycol); PEG-PHDCA, poly(PEGcyanoacrylate-co-hexadecylcyanoacrylate).

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ing Gram-positive and Gram-negative bacteria, spore-forming bacteria, and yeast and fungi.

This has been investigated in this study by employing high hydrostatic pressure (HHP) treatment to sterilize nanoparticle suspensions.

The main advantage of HHP treatment is that it does not convey nearly as much energy as heat treatment or high-pressure homogenization (10), which should help preserve the integrity of the nanoparticles. Moreover, pressurization should not affect the chemical composition of polymer drug carriers, as covalent bonds have a low compressibility and are, therefore, much less sensitive to changes in pressure (11). Finally, HHP acts instantaneously and uniformly throughout the whole product, independent of its size, shape, composition, and consistency. On the other hand, HHP is known to allow the inactivation of multiple vegetative microorganisms in hydrated samples (12). This has been widely documented in food science where commercial pressurized food products (fruit juices, seafood) displayed an extended shelf life due to microbial sanitation while remaining unaltered and "fresh-like" after HHP treatment (11,13,14).

As a first step toward using HHP as a new and safe way to sterilize thermally or hydrolytically labile nanoparticulate drug carriers, this study assessed the influence of such treatment on the physicochemical characteristics of thermosensitive poly(cyanoacrylate) nanoparticles. Moreover, a microbiological test was performed, to verify that the pressurized product complies with the pharmacopoeial requirements for sterility.

MATERIALS AND METHODS

Materials

Chemicals

Nanoparticles (nanospheres or nanocapsules) were prepared using poly(hexadecylcyanoacrylate) (PHDCA) polymer (conventional nanoparticles) or poly(MePEGcyanoacrylate-co-hexadecylcyanoacrylate) 1:4 (PEG-PHDCA) copolymer (sterically stabilized nanoparticles). The synthesis of these two polymers is described elsewhere in details (15). To confer a protective cloud to the conventional PHDCA nanospheres, the triblock amphiphatic polymer Pluronic (Lutrol® F68, BASF, Ludwigshafen, Germany) was adsorbed at their surface. The internal oily phase of the PHDCA and the PEG-PHDCA nanocapsules was Miglyol® (Miglyol 812N, Condea Chemie GmbH, Witten, Germany). Bi-distilled water was used as the dispersion medium for the nanoparticles (MilliQ water, Millipore). All other compounds were of analytical grade.

High Pressure Equipment

The system presented in Fig. 1, and located in the Laboratory of High Pressures Physical-Chemistry, (ENSCPB) was designed and produced by NFM-Technology and Framatome. It was marketed by Clextral. The equipment consisted of: a 3L water-filled cylindrical vessel (thermally insulated), one end closure and a hydraulic jack for restraining the vessel volume, a low pressure pump, an intensifier that used liquid from the low pressure pump to generate HHP process fluid for system compression, and control systems and instrumentation. The working pressure range for this machine was 0.1 to 800 MPa. The temperature inside the vessel (working interval: –20°C–80°C) was controlled using a UKS 3000 Lauda thermal flux generator coupled to the hydrostatic press. During the experiments, time, pressure and temperature inside the enclosure, hydraulic pressure, and the position of the hydraulic jack, were continuously recorded by a real time acquisition data system (NFM) and monitored by specific software (Visual Basic program).

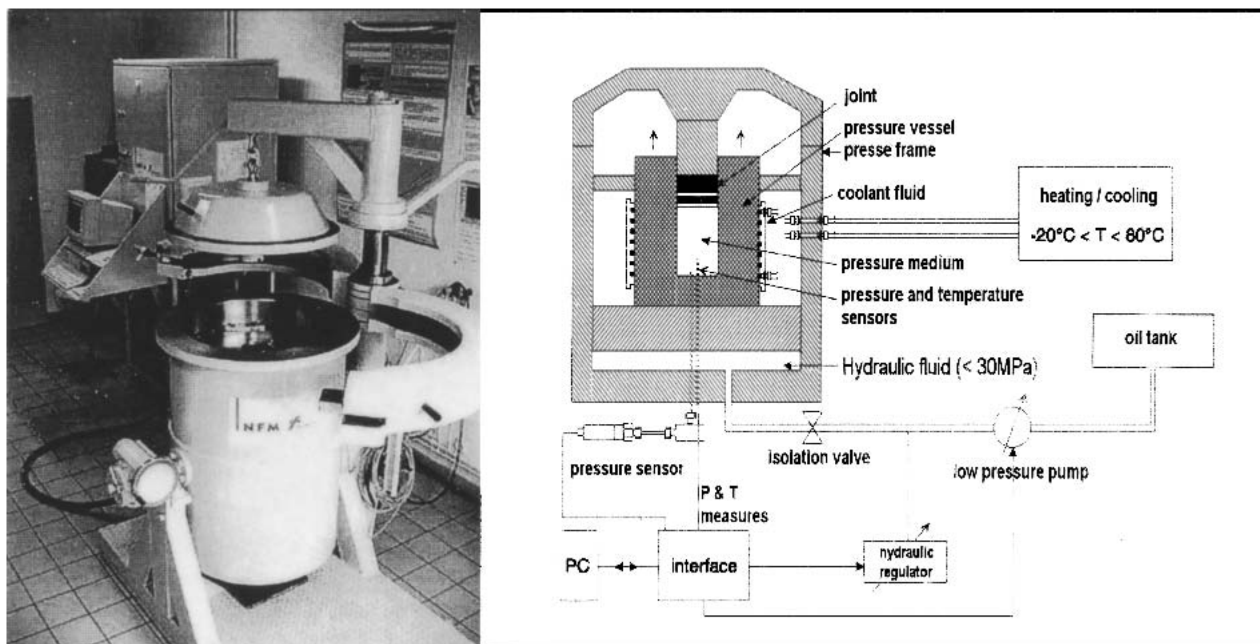

Fig. 1. High hydrostatic pressure (HHP) equipment (3-liter water-filled enclosure, 800 MPa, –20°C–+80°C). From the laboratory of Physicochemistry and High Pressures (ENSCPB), Bordeaux. *Equipement realized by FRAMATOME - NFM-Technology.*

Methods

Nanoparticles

In this study, the term "nanoparticles" is used to designate submicronic (<1 μm) polymer colloidal systems without specification regarding their morphology. The term "nanospheres" refers to nanoparticles with a matricial morphology, whereas "nanocapsules" are vesicular systems with an oily core surrounded by a thin polymer membrane.

To screen the influence of pressurization on the type of nanoparticles (nanospheres or nanocapsules), on the method of preparation (nanoprecipitation (16) or emulsion/solvent evaporation (17)), on the nanoparticle surface properties (conventional or sterically stabilized surface), and finally on the type of surface grafting in case of sterically stabilized colloids (covalent linkage or single adsorption), seven batches of nanoparticles (5 mg/mL, 90 mL) were prepared:

- PHDCA nanospheres obtained by nanoprecipitation (PHDCA np)
- PHDCA nanospheres obtained by emulsion/solvent evaporation (PHDCA ee)
- PHDCA nanocapsules obtained by nanoprecipitation, by adding Miglyol in the organic phase (1.7% v/v) (PHDCA nc)
- PHDCA nanospheres obtained by nanoprecipitation, with adsorbed Pluronic F68 (1% w/v in the aqueous phase) onto their surface (PHDCA np + F68)
- PEG-PHDCA nanospheres obtained by nanoprecipitation (PEG-PHDCA np)
- PEG-PHDCA nanospheres obtained by emulsion/solvent evaporation (PEG-PHDCA ee)
- PEG-PHDCA nanocapsules obtained by nanoprecipitation, by adding Miglyol in the organic phase (1.7% v/v) (PEG-PHDCA nc)

Details concerning nanoparticles preparation were described previously by Brigger *et al.* (15).

The nanospheres were purified by ultracentrifugation (145000 g, 1h30, 4°C, Beckmann L7-55 ultracentrifuge, USA). The pellets were resuspended in 90 mL of milliQ water to achieve a concentration of 5 mg/mL of precipitated material. No washing step was performed for the nanocapsules.

Afterwards, on a nanoparticle batch of 90 mL, 10 mL was kept as reference (control sample). The other 80 mL were divided into 8 fractions of 10 mL; each fraction was then subjected to a specific HHP cycle. On each sample of 10 mL (pressurized or not), 5–6 mL were freeze-dried (24h, −30°C/ +20°C, Christ Lyophilisateur alpha 1-4 (lock-1), Bioblock Scientific) for analysis on the solid state (infrared and scanning electron microscopy) (the PHDCA nanocapsule batch was unstable during freeze-drying, impeding studies on the solid state). The remaining 4–5 mL were kept for analysis on the liquid state (size measurement, zeta potential, turbidimetry) and maintained at 4°C.

High Pressure Processing

The process cycle for HHP sterilization consisted of charging the vessel with the product, priming the system, bringing the vessel to pressure process and temperature conditions, decompressing the vessel, and removing the product. Practically, 10 mL of each nanoparticle sample were sealed in watertight poly(ethylene)-polyamide bags before HHP treatment. Compression and decompression steps were performed at a rate of 100 MPa/min. To test the integrity of the nanoparticle suspensions during HHP treatment, the experimental pressures used were of 200 MPa, 300 MPa, 400 Mpa, and 500 MPa. Pressure holding-times were either 10 or 30 min. Finally, the working temperature was set at 25°C.

Analysis of Nanoparticles

Turbidimetry Measurements. After appropriate dilution in milliQ water of the nanoparticle suspensions, submitted or not to HHP, transmittance measurements were carried out by using a λ11 Perkin-Elmer UV/VIS spectrometer. A wavelength of 400 nm was selected.

Size Measurements. The hydrodynamic mean diameter of the nanoparticles and their size distribution were determined in milliQ water, at a 90° angle, by quasi-elastic light scattering (Coulter® N4MD, Coulter Electronics, Hialeah, USA). The analysis was done in triplicate, at 20°C. The size of the nanoparticle samples, treated or not by HHP, was measured after completion of the process as well as 30 days later (the stability test was performed on the control, the 200 MPa-10 min and the 500 MPa-30 min samples).

Zeta Potential Measurements. The surface charge of the nanoparticle suspensions, pressurized or not, was measured on a zetasizer (Zeta Sizer 4, 7032 Multi 8 Correlator, Malvern Instrument). Samples were analyzed with the no layer method (zeta advanced program), after appropriate dilution with NaCl 1 mM.

Scanning Electron Microscopy. The morphology and the size of the freeze-dried samples (consisting in the control and the 500 MPa-30 min samples), coated with a 6 nm Platinum-Palladium layer, were analyzed by using a scanning electron microscope equipped with a Gemini column (LEO 9530, Rueil-Malmaison, France).

Infrared Analysis. Infrared spectra of nanoparticle samples before and after HHP treatment were recorded on an interferometer based Brücker Vector 22 spectrometer, in the range of 4500–550 cm^{-1}, at a 1.92 cm^{-1} full resolution. Pretreatment by multiplicative scatter correction (MSC) and first derivative, as well as principal component analysis (PCA) were then performed on the infrared spectra as previously described, with The Unscrambler® software (Camo SA, Trondheim, Norway) (15).

Sterility

The sterility test was performed according to pharmacopoeial requirements (Ph. Eur., addendum 2002), on the purified PEG-PHDCA nanosphere suspension (5 mg/mL) prepared by nanoprecipitation.

To illustrate that the HHP technique would be suitable for a wide variety of microorganisms, the following species were used as indicators: *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633) and *Pseudomonas aeruginosa* (ATCC 33359) as aerobic bacteria; *Clostridium sporogenes* (ATCC 19404) as an anaerobic bacterium; *Candida albicans* (ATCC 90028) and *Aspergillus niger* as yeast and filamentous fungi, respectively.

For parenteralia preparations purporting to be sterile and containing less than 100 units in a batch, the minimum of units to be tested is 4. Hence, 4 bags containing 4 mL of the

nanosphere suspensions were soiled with a minimum of 10^3 colony forming unit (cfu) of each type of microorganism. This concentration was chosen, because it matched an important bacterial contamination in a nanoparticle suspension prepared and redispersed in sterile water. Samples were then sterilized by oscillatory pressurization (6 × 500 MPa consecutive HHP cycles of 5 min, at 25°C). Moreover, 4 reference units, without added microorganism, were sterilized according to the same procedure, to evaluate the effectiveness of the treatment on a normal microorganisms bioburden present after nanospheres preparation.

The sterility test was performed afterwards, according to the procedure of direct transfer to test medium. The sterility test, as well as the preliminary experiments (growth promotion test—bacteriostasis and fungistasis), used liquid media Buffered Dextrose broth (10 mL, BioRad, Marne-La-Coquette, France), and thioglycollate resazurcine broth for *C. sporogenes* detection (AES, Laboratoire, Combourg, France). All media were then incubated at 30°C for 14 days and examined regularly for macroscopic trouble apparition. Moreover, the number of bacteria (cfu) in the samples was determined by seeding appropriate dilutions on trypticase soy agar plates supplemented with 5% horse blood (BioMérieux, Marcy L'Etoile, France).

Preliminary experiments were performed to ascertain the growth of all tested microorganisms in the different inoculation media (growth promotion test) and to exclude any antimicrobial effect due to PEG-PHDCA nanospheres (bacteriostasis and fungistasis test). The nanospheres integrity and morphology were also checked for the applied pressurization cycle, as described earlier.

RESULTS

Analysis of Nanoparticles

Turbidimetry Measurements

No visible change was observed in the different nanoparticle samples, before and after pressurization, with respect to sedimentation, flocculation, aggregation, color, and loss of the oily content in the case of nanocapsules. Hence, a more precise analysis was performed by turbidimetry at 400 nm, to monitor size variations or solubilization/aggregation processes (18). As represented in Fig. 2, HHP treatment did not significantly affect the nanoparticle samples, because their observed optical density was generally within +/− 5% of that reported for the corresponding control. Moreover, there appeared to be no clear correlation between the turbidimetric behavior and the pressure applied to the nanoparticules and/or the time of compression, except for the PHDCA nanospheres coated with Pluronic F68, which featured a pressure-related gradual increase in optical density.

Size Measurement

All control samples showed an unimodal size distribution. HHP treatment did not affect the mean nanoparticles size (unimodal analysis depicted in Fig. 3) and size distribution (plurimodal analysis): neither a size reduction, nor the apparition of a new class of particles were observed, except for the PHDCA batch coated with Pluronic F68. In this case, the mean diameter increased by 10 nm with the application of

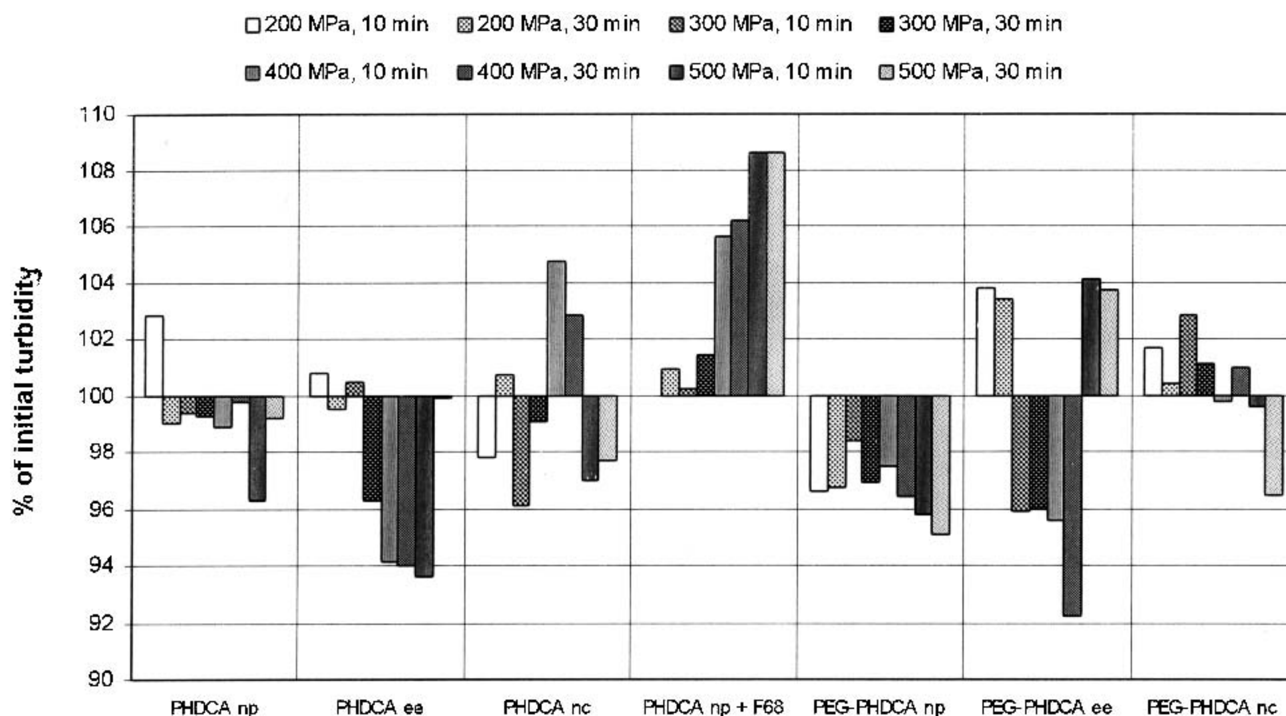

Fig. 2. Percentage of variations in the turbidity (at 400 nm of wavelength) of the pressurized samples, when compared to the non pressurized control sample (100%). Analysis of 7 colloidal batches: PHDCA np or PEG-PHDCA np: PHDCA or PEG-PHDCA nanospheres obtained by nanoprecipitation; PHDCA ee or PEG-PHDCA ee: PHDCA or PEG-PHDCA nanospheres obtained by emulsion/solvent evaporation; PHDCA nc or PEG-PHDCA nc: PHDCA or PEG-PHDCA nanocapsules obtained by nanoprecipitation; PHDCA np + F68: PHDCA nanospheres with adsorbed Pluronic F68 onto their surface.

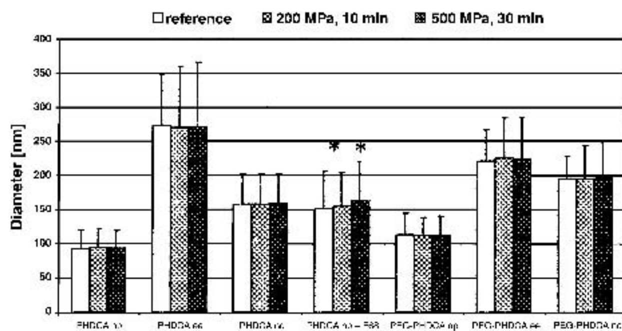

Fig. 3. Unimodal size distribution and standard deviation from 3 measurements on following samples: control (unpressurized sample), HHP 200 MPa–10 min and HHP 500 MPa–30 min (other pressurized samples not represented). Analysis of 7 colloidal batches: PHDCA np or PEG-PHDCA np: PHDCA or PEG-PHDCA nanospheres obtained by nanoprecipitation; PHDCA ee or PEG-PHDCA ee: PHDCA or PEG-PHDCA nanopsheres obtained by emulsion/solvent evaporation; PHDCA nc or PEG-PHDCA nc: PHDCA or PEG-PHDCA nanocapsules obtained by nanoprecipitation; PHDCA np + F68: PHDCA nanospheres with adsorbed Pluronic F68 onto their surface. *: samples displaying a plurimodal size distribution.

400 and 500 MPa pressures. Moreover, pressurization of these nanospheres generally induced a bimodal size distribution, with a class ranging between 50–100 nm and another between 200–300 nm.

The 1-month stability test (data not shown) carried out on each colloidal formulation presented a maximum nanoparticles size increase of 10%. However, HHP treatment did not accelerate this instability.

Zeta Potential

From the results reported in Fig. 4, conventional and sterically stabilized nanoparticles (by adsorption with Pluronic F68 or covalent binding with PEG) displayed similar surface potential before and after HHP treatment. A maximum difference of -6 mV between the control samples and certain HHP treated samples was observed (PHDCA nanocapsules and PEG-PHDCA nanospheres obtained by emulsion/solvent evaporation). These differences, however, were small and could neither be correlated with the applied pressure nor with the pressure-holding time.

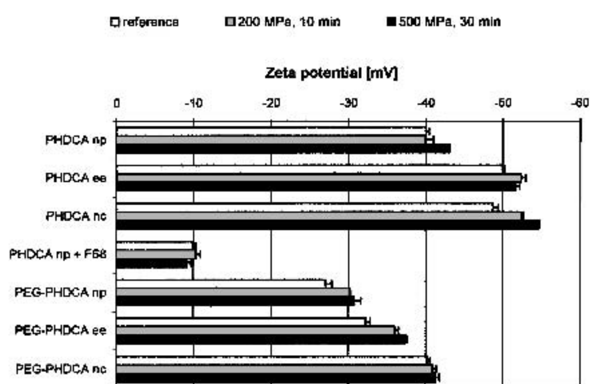

Fig. 4. Zeta potential and standard deviation from 3 measurements on following samples: control (unpressurized sample), HHP 200 MPa–10 min and HHP 500 MPa–30 min (other pressurized samples not represented). Analysis of 7 colloidal batches: PHDCA np or PEG-PHDCA np: PHDCA or PEG-PHDCA nanospheres obtained by nanoprecipitation; PHDCA ee or PEG-PHDCA ee: PHDCA or PEG-PHDCA nanopsheres obtained by emulsion/solvent evaporation; PHDCA nc or PEG-PHDCA nc: PHDCA or PEG-PHDCA nanocapsules obtained by nanoprecipitation; PHDCA np + F68: PHDCA nanospheres with adsorbed Pluronic F68 onto their surface.

Scanning Electron Microscopy

Scanning electron microscopy pictures of the PEG-coated nanospheres obtained by nanoprecipitation are presented in Fig. 5 (top). This picture was representative of all the analyzed nanoparticle batches. Observations showed that continuous HHP treatment at 500 MPa during 30 min did not alter the size or the morphology of the nanoparticles, as compared with the non-pressurized control sample.

Infrared Analysis

Infrared spectra evidenced no change in the chemical structure of each nanoparticle batch after HHP processing. No shifts, or new peaks were noticed, except a weak band at 3666 cm^{-1} assigned to water residue in the pressurized samples of PHDCA nanospheres coated with Pluronic F68 and PEG-PHDCA nanocapsules (Fig. 6).

For each nanoparticle batch, infrared spectra presented a base-line offset, due to measurement variability. To correct this offset and minimize intra-batch variability, spectra transformation was performed by two pre-treatments. PCA was then performed on the pretreated infrared spectra of all the nanoparticle batches included in this study. The underlying idea in PCA modeling is to replace a complex multidimensional data set (i.e., the spectra described by their reflectance value at each wavenumber) by a simpler version involving fewer dimensions but still fitting the original data close enough to be considered a good approximation (the spectra treated with PCA are described by their score on each loading in the principal component (PC) space). By plotting the two or three first loadings (loadings describe the relationships between variables), similar spectra cluster in the same region of the PC space, whereas compounds with spectral differences cluster in other parts of this space. As presented in Fig. 6E, PCA allowed the separation of the different nanoparticle batches mainly according to their chemical composition (content in ethyl and ether functions). Moreover, each nanoparticle batch, processed or not by HHP, displayed a well-defined cluster without important intra-class variability. Hence, as no outlier was noticed for the different batches, even for the PEG-PHDCA nanocapsules and the PHDCA nanospheres coated with Pluronic F68, this suggested that HHP treatment did not influence the chemical structure of the compounds involved in the formulations (PHDCA, PEG-PHDCA, Pluronic F68 and Miglyol®).

Sterility Testing

First, the sterilization method by HHP treatment (6 cycles of 5 min at 500 Mpa) was validated before, on highly contaminated samples (>10^6 cfu) of Gram+ and Gram- vegetative bacteria (Y. Rigaldie, personal communication and thesis n° 2526, University of Bordeaux I, France). Then, to test the feasibility of sterilizing thermo-sensitive PEG-PHDCA drug carriers by HHP treatment, nanosphere

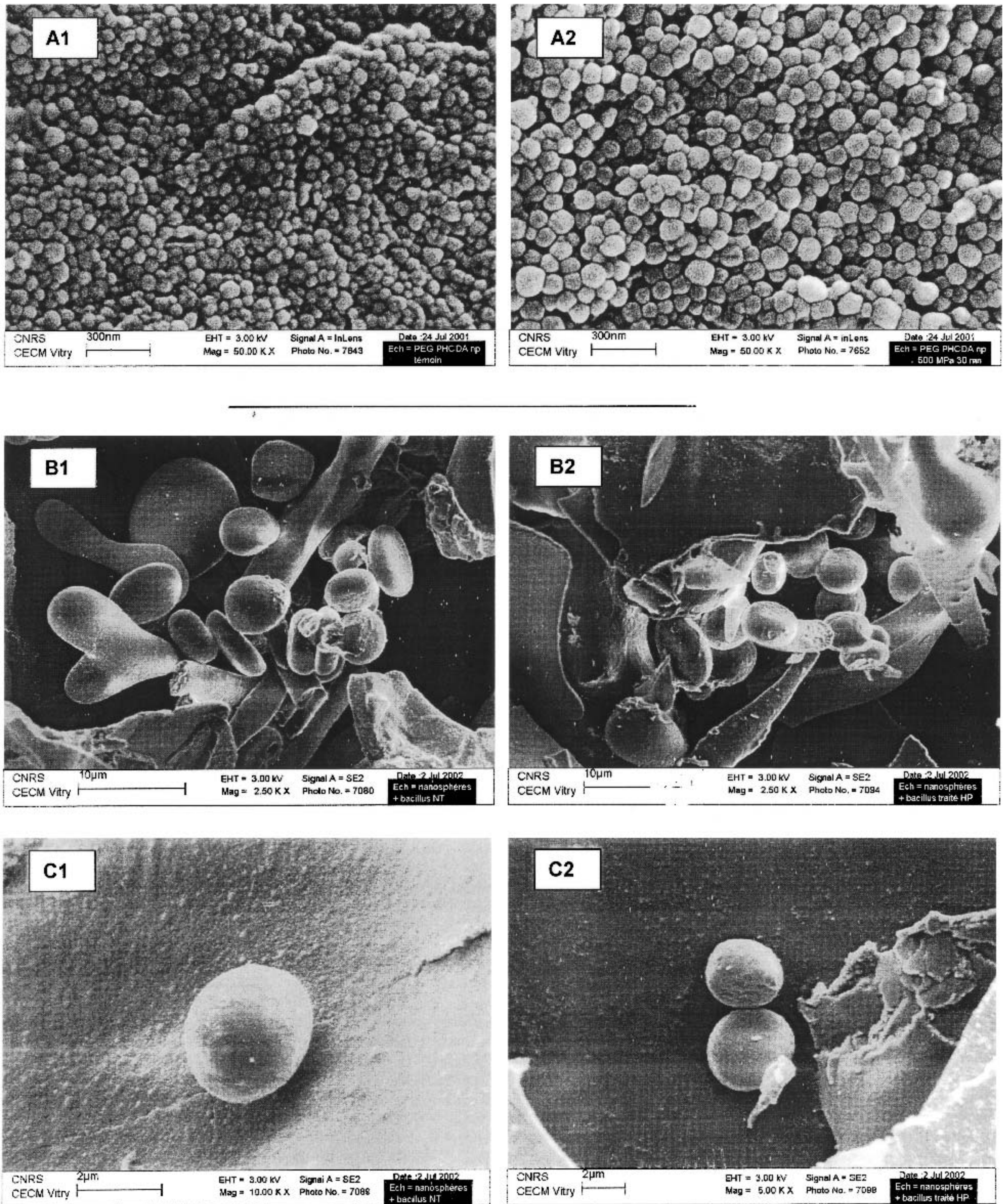

Fig. 5. PEG-PHDCA nanospheres obtained by nanoprecipitation submitted (A2) or not (A1) to a continuous 500 MPa HHP treatment (30 min) (Top). PEG-PHDCA nanospheres containing spore or spore-forming *Bacillus subtilis* submitted (B2 and C2) or not (B1 and C1) to an oscillatory 500 MPa HHP treatment (30 min) (Bottom). Nanospheres are regrouped in plaques during freeze-drying; they become distinguishable in the background of C1 and C2 pictures.

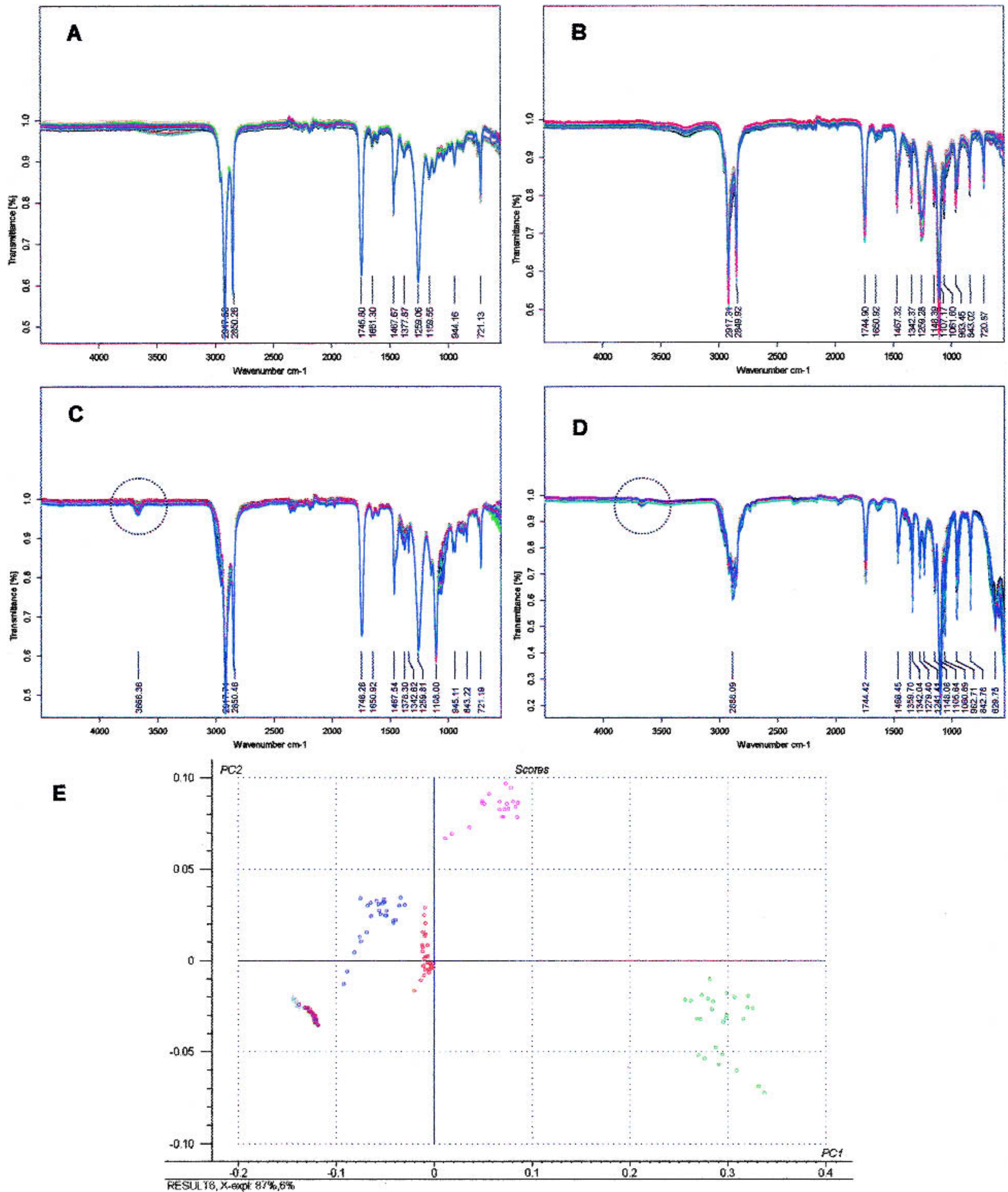

Fig. 6. Superimposed typical infrared spectra of samples, submitted or not to the different HHP treatments, of (A) PHDCA np; (B) PEG-PHDCA np; (C) PHDCA np + F68, as well as (D) PEG-PHDCA nc. (other batches not represented). [Identification of functional groups: cyano group (weak): 2300–2000 cm^{-1}; carboxylic group: 1745 and 1259 cm^{-1}; ether: 1107 cm^{-1}, alkyl groups: CH_3: 2917, 2850 and 1377 cm^{-1}, CH_2: 2917, 2850 and 1467 cm^{-1}]. (E) PCA performed on the MSC and I derivative pretreated infrared spectra of following nanoparticle batches, pressurized or not: PHDCA np (grey) or PEG-PHDCA np (pink): PHDCA or PEG-PHDCA nanospheres obtained by nanoprecipitation; PHDCA ee (brown) or PEG-PHDCA ee (blue): PHDCA or PEG-PHDCA nanopsheres obtained by emulsion/solvent evaporation; PEG-PHDCA nc (green): PEG-PHDCA nanocapsules obtained by nanoprecipitation; PHDCA np + F68 (red): PHDCA nanospheres with adsorbed Pluronic F68 onto their surface.

samples were spoiled with approximately 10^4 cfu of biomaterials (important degree of contamination for a nanoparticulate suspension prepared with sterile water). The results presented in Table I, showed a general reduction of 3–4 log of microorganisms number on completion of product conditioning, leading sterile samples, in the case of non-sporulating bacteria. Moreover, non HHP-treated reference nanosphere samples (without added microorganisms) were slightly contaminated (<50 cfu/bags). Microorganisms were identified as *Staphylococcus sp* and *Micrococcus sp*. However, after HHP processing, these samples displayed no microbial growth.

Scanning electron microscopy performed on the samples of PEG-coated nanospheres containing the different microorganisms revealed no differences before and after oscillatory HHP processing at 500 MPa. For the microorganisms, neither rupture of their cell wall/membrane nor size and shape variations were evidenced (Fig. 5, bottom, with *B. subtilis* as example). Precise information on the surface of microbes was hardly accessible, as the nanospheres tended to form a deposition layer during freeze-drying. For spore-forming microorganisms like *B. subtilis* (Fig. 5, bottom) and *C. sporogenes*, spores were still present in the samples after pressurization. On the other hand, variations in the nanospheres with respect to size and morphologic appearance, turbidity, as well as surface charge were not observed (data not shown).

DISCUSSION

Already used in the food industry as a way to inactivate microorganisms and enzymes (13), HHP processing was tested in this study for the sterilization of heat labile pharmaceutical colloidal carriers.

A variety of thermosensitive poly(cyanoacrylate) nanoparticle suspensions (nanospheres and nanocapsules, with or without surface modification) were submitted to different HHP treatment conducted at 25°C: continuous pressurization at 200, 300, 400, or 500 MPa during 10 or 30 min, as well as oscillatory pressurization with six 5-min cycles at 500 MPa (total pressure holding time: 30 min).

The data obtained demonstrated that all the tested HHP treatments induced no physical damage on the different nanoparticle suspensions, except for the Pluronic F68- coated PHDCA nanosphere batch. For the other batches, even the more fragile nanocapsules, no significant variation of the visual appearance, morphologic structure, mean diameter, and size distribution were reported after pressurization. These observations were confirmed by the turbidity experiment, where the reduction in light transmission at 400 nm caused by light scattering depends on nanoparticles size and concentration (18). The optical density of the HHP treated samples was close to that of the control sample, suggesting again that neither size variation, nor solubilization/aggregation processes occurred during HHP treatments. With regard to surface charge, maximal differences of −6 mV comparatively to the reference were measured. Such slight variations, however, did not have any consequence on the colloidal sample stability, as displayed by the 1-month stability test, during which time no aggregation/flocculation occurred (only Ostwald ripening). Moreover, according to infrared analysis, no chemical damage was evidenced on these nanoparticle batches.

For Pluronic-coated PHDCA nanospheres, a pressure-related instability was revealed by size and turbidity measurements. However, as uncoated PHDCA nanospheres displayed no physical variation under HHP treatment, the observed swelling effect and plurimodal distribution was probably due to the adsorbed Pluronic coating. Although chemical damage on the pressurized Pluronic F68 samples was not evidenced by infrared analysis, the presence of water in the infrared spectra could explain destabilization of the coating layer under HHP. This was clearly not the case with covalently attached PEG at the surface of nanospheres, which displayed no size variation after HHP treatment. This suggests that HHP sterilization may be applied to nanoparticles decorated with chemically bound macromolecules, because it is accepted that covalent bounds are resistant to high pressures (11). On the contrary, when polymers are only adsorbed on the nanoparticles surface, HHP treatment should be applied with greater caution because layer destabilization may occur.

Altogether, these results emphasize that HHP did not modify physically or chemically poly(alkylcyanoacrylate) nanospheres, and nanocapsules, regardless of their preparation method and their surface characteristics (however, precautions should be taken in case of adsorbed surface modifiers). Under pressure, poly(cyanoacrylate) nanoparticles seemed to behave like perfect elastic compounds (19).

Nevertheless, future work should investigate whether HPP can successfully sterilize nanoparticles containing an encapsulated drug and check drug integrity after such a treatment. This investigation is especially important for encapsulated proteins like insulin (or even for gelatin nanoparticles), because HHP is known to denature proteins (20). In addition, drugs adsorbed at the nanoparticles surface by ion pairing

Table I. The Effect of HHP Processing on the Inactivation of Various Microorganisms Present in the Dispersion Medium of PEG-PHDCA Nanospheres

Samples[a]	cfu/4 mL nanospheres suspension before HHP sterilization	cfu/4 mL nanospheres suspension after HHP sterilization[b]
nanospheres + Staphylococcus aureus (ATCC 25923)	5×10^4	0
nanospheres + Pseudomonas aeruginosa (ATCC 33359)	3×10^4	0
nanospheres + Bacillus subtilis (ATCC 6633)	5×10^4	positive (cfu: nd)
nanospheres + Clostridium sporogenes (ATCC 19404)	4×10^4	n.d.
nanospheres + Candida albicans (ATCC 90028)	2×10^4	0
nanospheres + Aspergillus niger	10^3	0
nanospheres as prepared	2/4 units positive (cfu: nd)	0

[a] For practical reasons, the PEG-PHDCA nanospheres samples (5 mg/mL) were kept at 4°C 20 h before and after HP sterilization.
[b] HP sterilization cycle: 6 consecutive cycles at 500 Mpa; pressure holding time of a cycle: 5 min.

(for example oligonucleotides on positively charged colloids), could be desorbed, as it is generally admitted that HHP induces the breakdown of salt bonds due to electrostriction (11). Moreover, questions may arise in case of more complex constructs like antibody-functionalized nanoparticles. Hence, the limits of HHP treatment on polymeric systems are yet to be defined.

For sterilization of PEG-PHDCA nanospheres, an oscillatory 500 MPa-HHP cycle at 25°C (6 × 5 min) was chosen, as Gram+ bacteria, especially *S. aureus*, are known to have a high resistance to continuous pressure (21).

In the preliminary tests performed to validate the sterility test, even though the cyanoacrylate monomer is reported to be a strong bactericidal agent (19), PEG-decorated poly-(hexadecylcyanoacrylate) nanospheres did not slow down microorganisms growth. Moreover, the nanoparticles dispersion medium was free of isotonizing agents, which could have a baroprotective effect for microorganisms (11). Thus, the observations made in the sterility test only resulted from the effect of HHP on microorganisms.

The sterility test performed according to pharmacopoeial requirements on contaminated nanoparticle samples demonstrated that oscillatory HHP treatment allowed to obtain sterilized batches of PEG-PHDCA nanospheres, when contaminated with vegetative Gram+ and Gram− bacteria, yeast, and fungi. However, observations of all tested microorganisms by scanning electron microscopy illustrated that neither morphologic changes, nor defect of the cells were observed after HHP processing. This suggests that the underlying sterilization mechanism for vegetative microbes is not by cell rupture. High hydrostatic pressure has been reported to interfere with various cellular structures or functions (13,22). For example, the bilayer of the plasma membrane is ordered by pressure, which may cause the detachment of peripheral or integral proteins (22). Depolymerization of cytoskeletal proteins may also play a role in the inactivation mechanism (22). Moreover, various microbial enzymatic systems can be denatured under high pressure, such as the Na/K-dependent ATPase that is located in the phospholipid bilayer and involved in active transport phenomena through the membrane or the F0F1 ATPase. In this later case, without energy normally supplied by ATP hydrolysis, active transport out of the cell cannot take place, and the cell dies due to acidification (23). Finally, apart from the cytoplasmic membrane, there are other targets for HHP inactivation like ribosomes and soluble enzymes (10,23).

Bacterial spores (i.e., *B. subtilis* and *C. sporogenes*), were present in the samples after HHP sterilization according to the pictures obtained by electron microscopy. Now, spores are extremely baroresistant and only sublethally injured by the sterilization conditions used in this study. As their viable number can only be reduced in combination with high temperature (70°C) (increase of water permeability) (13,24) or under very high pressure conditions (up to 1000 MPa) (adiabatic explosion of spore cells) (13,23), spores are still cause for concern, because they can contaminate the samples when external conditions are favorable for germination. This fact was demonstrated by the positive results obtained for *B. subtilis* in the sterility test. This result emphasizes the need to develop effective non-thermal protocols with a two-exposure HHP treatment: the first exposure at low pressure germinates or activates the spores, while the second exposure at a higher pressure inactivates the germinated spores and vegetative cells (similar schema as tyndallization by heat) (13,25).

In conclusion, this work proposes HHP treatment as a soft and non-thermal sterilization technique for labile polymeric nanoparticle suspensions, providing that better protocols are developed, to efficiently eliminate sporulating bacteria. Nevertheless, such new protocols are already under investigation and allowed a 10^7-cfu reduction of *B. subtilis*, without excessive heating (Y. Rigaldie, personal communication and thesis n°2526, University of Bordeaux I, France). In addition, PEG-coated cyanoacrylate nanospheres showed extreme high resistance to these long HHP processes.

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