J Mater Sci: Mater Med (2015) 26:32
DOI 10.1007/s10856-014-5362-6

One-step formation and sterilization of gellan and hyaluronan nanohydrogels using autoclave

Elita Montanari · Maria Cristina De Rugeriis · Chiara Di Meo · Roberta Censi · Tommasina Coviello · Franco Alhaique · Pietro Matricardi

Received: 7 April 2014 / Accepted: 12 September 2014 / Published online: 13 January 2015

Abstract The sterilization of nanoparticles for biomedical applications is one of the challenges that must be faced in the development of nanoparticulate systems. Usually, autoclave sterilization cannot be applied because of stability concerns when polymeric nanoparticles are involved. This paper describes an innovative method which allows to obtain, using a single step autoclave procedure, the preparation and, at the same time, the sterilization of self-assembling nanohydrogels (NHs) obtained with cholesterol-derivatized gellan and hyaluronic acid. Moreover, by using this approach, NHs, while formed in the autoclave, can be easily loaded with drugs. The obtained NHs dispersion can be lyophilized in the presence of a cryoprotectant, leading to the original NHs after re-dispersion in water.

1 Introduction

During last decades, interest in polymeric nanoparticles has tremendously increased because of the wide spectrum of applications that such structures may offer. Their uses range from medicine to pharmaceutics, diagnostics and biotechnology, from sensors to conducting materials and catalysis, as well as electronics, photonics, food/enzyme stability, pollution control, etc. [1, 2]. Many macromolecules leading to such nanostructures have been proposed and several techniques can be adopted for their preparation [3]. Among the polymers that can be used within the field of biomedical applications, polysaccharides are particularly suitable since they are abundant in nature and readily available from renewable sources and have a large variety of composition and properties (e.g. biodegradability) that cannot be easily mimicked by synthetic macromolecules. Furthermore, due to the ease of their production, they are often cheaper than other types of polymers [4]. As far as the methods of preparation are concerned, polymeric nanoparticles can be obtained either by appropriate aggregation of macromolecules (e.g., nanoemulsions, nanoprecipitation, solvent evaporation, dialysis, salting out, sonication, supercritical CO_2), or by means of the monomer polymerization techniques (as in the case of dendrimers). When these nanostructures are used as drug carriers in pharmaceutical administration, they must be sterilized (injectable preparations, ocular applications) and the procedures that usually can be adopted (filtration, autoclaving, etc.) are not always easily feasible because of nanoparticles stability concerns [5]. Actually, the physicochemical properties of pre-formed loaded nanoparticles can be significantly affected by the high temperature and pressure conditions of an autoclave or by the high energy due to the γ irradiation; at the same time, filter sterilization is often not adequate for such structures, from a technological point of view, because of gradual pore filter plugging.

Here we offer an innovative method for the preparation of gellan and hyaluronan self-assembling nanohydrogels (NHs) which allows to obtain, using an autoclave and in a single step, self-assembling of the macromolecules and their sterilization. The same were previously prepared by sonication and extensively studied for pharmaceutical applications [6, 7]. The use of the autoclave can thus lead

E. Montanari · M. C. De Rugeriis · C. Di Meo · T. Coviello · F. Alhaique · P. Matricardi (✉)
Department of Drug Chemistry and Technologies, "Sapienza" University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy
e-mail: pietro.matricardi@uniroma1.it

R. Censi
School of Pharmacy, University of Camerino, Via S. Agostino, 1, 62032 Camerino, MC, Italy

directly to sterile NHs; moreover, during the same procedure, these nanoparticles can be loaded with hydrophilic and lipophilic drugs. It should also be pointed out that these nanostructures in the form hydrogels are especially suitable for biomedical applications because of the high water content which usually assures good biocompatibility and easy cell internalization [8–10].

2 Materials and methods

2.1 Materials

Hyaluronan tetrabutylammonium salt (HATBA, Mw = 1.5 × 10^5 according to the supplier) and gellan gum tetrabutylammonium salt (GeTBA, Mw = 2.5 × 10^6 according to the supplier) were kindly provided by Fidia Advanced Biopolymers, Abano Terme (PD), Italy. Cholesterol (CH), 4-bromobutyric acid, levofloxacin (LVF), *N*-methyl-2-pyrrolidone. (NMP), *N*-(3-dimethylaminopropyl)-*N*′-(ethylcarbodimide hydrochloride) (EDC·HCl), 4-(dimethylamino)pyridine (DMAP), *N*-hydroxysuccinimide (NHS), dextrose, tetramethylammonium chloride (TMA^+Cl^-), tetrabutylammonium bromide (TBA^+Br^-) were Sigma products.Other chemicals were reagent grade and were used without further purification.

2.2 Polysaccharides Mw analysis and gellan gum controlled depolymerization

The molecular weight (Mw) and polydispersity index (PDI) of gellan and hyaluronan were determined by gel permeation chromatography (GPC, Model 210—VARIAN). GeTBA was dissolved (0.15 % w/v) in 25 mM TMA^+Cl^- aqueous solution. The elution was then performed on two PL Aquagel–OH Mixed-H columns (8 μm, 300 × 7.5 mm) using 25 mM TMA^+Cl^- as eluent, at a flow rate of 1 mL/min [7]. HATBA was dissolved (0.15 % w/v) in 10 mM TBA^+Br^- aqueous solution; the elution was then performed using the same columns and 10 mM TBA^+Br^- as eluent.

A calibration curve based on pullulan (range of Mw 5900–788 000) in TMA^+Cl^- (25 mM) was used. GPC data were analyzed by means of Cirrus software. Obtained values were: Mw = 2.5 × 10^6 with a PDI of 10 for gellan and Mw = 1.5 × 10^5 with a PDI of 1.7 for hyaluronan.

The depolymerization of gellan gum tetrabutylamonium salt (GeTBA) was carried out according to a method previously described [7]. Briefly, GeTBA (0.5 g) was dissolved in distilled water (100.0 mL) at 80 °C; 50.0 mL aliquots of this solution were sonicated using a probe type sonicator (Vibra Cell—VC 750, microtip 6.5 mm), 20 kHz, at 30 % of amplitude for 60 min. To reduce the temperature increase, the solutions were kept in an ice bath and a pulsed mode (50 % sound–50 % silence) was applied. The solutions were then filtered (0.22 μm, Millipore filter) and dialyzed against distilled water (Visking tubing, cut-off 12,000–14,000) until the conductivity reached a value below 1.2 μS. The pH of the dialyzed solution was adjusted to 7, using TBA^+OH^- (0.05 M) and the polymer was recovered by freeze-drying, using a "Modulyo 4 K" Edwards High Vacuum instrument, equipped with an Edwards pump, operating at 0.2 at. and at −40 °C. The viscometric Mw of GeTBA sonicated for 60 min was determined by GPC (obtained values: Mw = 2.3 × 10^5; PDI = 3.7).

2.3 Gellan-cholesterol and HA-cholesterol derivatives: synthesis and characterization

Gellan-cholesterol (GeCH) and HA-cholesterol (HACH) synthesis was carried out as previously described [6, 7].

First of all, cholesterol was derivatized with 4-bromobutyric acid leading to the Br-butyric-cholesterol (CH-Br) derivative.

GeCH was prepared by dissolving 100.0 mg of depolymerized GeTBA in 8.0 mL of *N*-methyl-2-pyrrolidone (NMP); the CH-Br derivative (6.0 mg), previously dissolved in 2.3 mL of NMP, was then added in order to obtain a GeTBA derivatization degree of 10 % mol/mol (mol of cholesterol per mol of Ge repeating units). The reaction was kept under magnetic stirring for 48 h at 38 °C [7]. HATBA (200.0 mg) was likewise dissolved in NMP (10.0 mL) and the CH-Br derivative (34.3 mg), solubilized in 2.0 mL of NMP, was added in order to obtain an HATBA derivatization of 20 % mol/mol (mol of cholesterol for mol of HA repeating units). The reaction was kept under magnetic stirring for 48 h at 38 °C [6].

Both preparations were exhaustively dialysed against distilled water and neutralized with TBA^+OH^- 0.1 M. GeCH and HACH were finally recovered by freeze-drying (yield ∼90 %).

2.4 One-step formation and sterilization of GeCH and HACH NHs

In a typical experiment 3.0 mg of each derivative (GeCH or HACH) were dispersed in 3.0 mL of distilled water (1.0 mg/mL) by overnight magnetic stirring at room temperature. The samples were then autoclaved for 20 min at 121 °C and 1.10 bar (Juno Liarre autoclave 230 Vac, 50/60 Hz, 12A, 2000 W), leading to NHs formation. The recovered GeCH NH and HACH NH suspensions were then analyzed for size and polidispersity (PDI) by means of dynamic light scattering (DLS) using Submicron Particle Sizer Autodilute Model 370 (Nicomp). NH suspensions

ζ-potential was measured with a DLS Malvern NanoZeta-Sizer apparatus (Malvern Instruments, Worcestershire, United Kingdom), equipped with a 5 mW HeNe laser ($\lambda = 632.8$ nm). Normalized intensity autocorrelation functions were detected at a 90° angle by a logarithmic digital correlator and analyzed by Contin algorithm.

The size and PDI stabilities of GeCH and HACH NHs suspensions in distilled water were evaluated at 4 °C for 1 month and at 37 °C for 1 week by means of DLS.

In a parallel experiment, 3.0 mg of each derivative (GeCH or HACH) were dispersed in 3.0 mL of dextrose aqueous solution (1 % w/v), used as cryoprotectant [6], autoclaved as described above, and immediately freeze-dried. The NH size and PDI were evaluated before and after the freeze-drying process and re-dispersion in water by means of DLS.

2.5 NHs Cryo-TEM

Suspensions of GeCH or HACH NHs (1.0 mg/mL, 3.0 mL) prepared as described above were ultracentrifuged (40,000 rpm, 4 °C, 3 h) using an ASHI WX ULTRA 80 Ultracentrifuge equipped with carbon fibre rotor, type fixed angle (Weight: 3.3 kg; volumetric capacity: 6 × 13.5 mL; maximum speed: 65,000 rpm; maximum RCF: 325,000). The obtained pellet was then re-suspended in 1 mL of distilled water (final concentration: 3.0 mg/mL) with a vortex mixer and analyzed by cryogenic transmission electron microscopy (CryoTEM). For the analysis, 4 μL of NHs suspension were laid onto a perforated carbon film mounted on a 200 mesh electron microscopy grid. Most of the drop was removed with blotting paper and the residual thin film, remaining within the holes, was vitrified after by immersion in liquid ethane using a guillotine-like frame. The specimen was then transferred, using liquid nitrogen, to a cryospecimen holder and observed with a JEOL FEG-2010 electron microscope. Micrographs were recorded at 200 kV under low-dose conditions at a magnification of 40,000 on SO-163 Kodak films. Micrographs were digitized using a film scanner.

2.6 One-step formation, drug loading and sterilization of GeCH and HACH NHs

GeCH or HACH (6.0 mg) were dispersed in 3.0 mL of distilled water. A LVF aqueous solution (3.0 mL, 0.66 mg/mL) was added to the polymer suspensions (polymer:LVF ratio 3:1) and the samples were autoclaved (121 °C, 1.10 bar, 20 min). LVF-loaded NHs (LVF-NHs) were purified from the unloaded levofloxacin by dialysis against distilled water for 3 h and then freeze-dried.

2.7 Encapsulation efficiency of GeCH and HACH NHs

The recovered freeze-dried products were completely dissolved in NMP (0.30 mg/mL). The LVF concentration was evaluated at $\lambda = 302$ nm with an UV–Vis spectrophotometer (Perkin-Elmer, double beam "Lambda 3A") using a linear calibration plot for LVF obtained over the range 0.75–12.0 μg/mL ($n = 5$, $R^2 = 0.999$) [11]. Drug encapsulation efficiency (i.e. % encapsulation, w/w) was determined as the ratio between the amount of the encapsulated LVF and the weight of dry NHs.

2.8 Improvement of the pre-formed GeCH and HACH NHs features by autoclaving

5.0 mg of GeCH and HACH polymers were dissolved in 5.0 mL of distilled water by overnight magnetic stirring at room temperature. For the NHs formation, the suspensions were sonicated for 25 min, using an ultrasonic bath sonicator (Strasonic-35, Liarre) [6, 7]. The NH suspensions size and PDI were analyzed by DLS instrument. NH suspensions were then autoclaved (121 °C, 1.10 bar, 20 min) and the NHs size and PDI stability were tested by DLS.

The size and PDI stability of the NH suspensions in water were evaluated at 4 °C for 1 month and at 37 °C for 1 week by means of DLS.

2.9 Statistical analysis of data

All the data presented in the manuscript are expressed as the mean ± standard deviation and were subjected to statistical analysis. Student's *t* test was used for comparison between samples and control. Significance was tested at the 0.05 level of probability (*P*).

3 Results and discussions

The aim of this work was to find a new simple method to form sterile NHs as well as drug-loaded sterile NHs, in a one-step procedure using the autoclave. Actually, autoclave is commonly used to sterilize medical devices and pharmaceutical preparations, which are stable at operating conditions (high pressure and high temperature). This condition is satisfied for many conventional preparations but it is scarcely applicable to nanoparticulate systems based on polymers because of their poor stability in such conditions. Amphiphilic polysaccharides, such as gellan-cholesterol [7] and hyaluronic acid-cholesterol [6], are polymeric systems that can form NHs by nanoprecipitation or by ultrasonication in water, as reported also in previous literature [9, 10]. Usually these procedures require many purification steps in order to eliminate chemicals and/or

impurities due to the NHs process formation. Moreover, the nanosuspensions, once prepared, must be sterilized by filtration (other sterilization processes are not suitable because of stability reasons) before administration. These technological steps are scarcely appealing in scaling up the NHs production as well as with low efficiency in product recovery (filter plugging). With the aim to generate, by means of a one step procedure, sterile nanoparticulate suspensions for administration in humans, we observed that by autoclaving a water suspension of gellan-cholesterol or hyaluronic acid-cholesterol, without any previous treatment, NHs are directly formed. Most probably, pressure and temperature conditions in the autoclave promote the onset of hydrophobic interactions between cholesterol moieties on the polysaccharide chains, leading to sterile NH suspensions with no need of other physico-chemical treatments.

The features of the HACH and GeCH NHs, obtained according to this new procedure, were perfectly comparable with those of NHs obtained by ultrasonication [6, 7] and nanoprecipitation (data not published), showing only a slight size increase in the case of HACH NHs.

GeCH NHs showed a mean size of 200 ± 10 nm, with a PDI of 0.20 ± 0.05, whereas HACH NHs showed a size of 360 ± 20 nm, PDI of 0.2 ± 0.05. ζ-potential values were −25 ± 5 and −30 ± 5 mV, respectively. As shown in Fig. 1, autoclave-formed NHs suspensions showed also good stability, both at 37 and 4 °C, for 1 month and 1 week, respectively.

Cryo-TEM analysis showed that HA-CH NHs have a spherical shape with a diameter of 100–130 nm (Fig. 2a), while GeCH NHs are smaller, and their diameter is about 70 nm (Fig. 2b).

In order to obtain a long-life preparation of NHs, thus improving the industrial appealing of such systems, an appropriate amount of a cryoprotectant solution, i.e. 1 % w/v dextrose, was added to the GeCH and HACH polymer dispersion, before the autoclaving process. The so formed GeCH and HACH NHs sterile preparations were directly freeze-dried and stored for several months.

As reported in Fig. 3, after freeze-drying and re-dispersion in water, HACH NHs showed only a slight increase in diameter (about 30 nm, $P < 0.01$), while GeCH NHs size increased up to 355 ± 15 nm ($P = 0.01$).

As above pointed out, the obtained NHs are suitable as carriers of both hydrophilic and lipophilic drugs. The mechanism underlying this property can be explained considering that the self-assembled NHs have hydrophobic moieties—the region responsible of the assembling phenomenon—and hydrophilic zones—due to the hydroxyl groups of the polysaccharide chains. Each of these regions can interact with molecules with different hydrophobicity or hydrophilicity. In particular, as evidenced in a recent

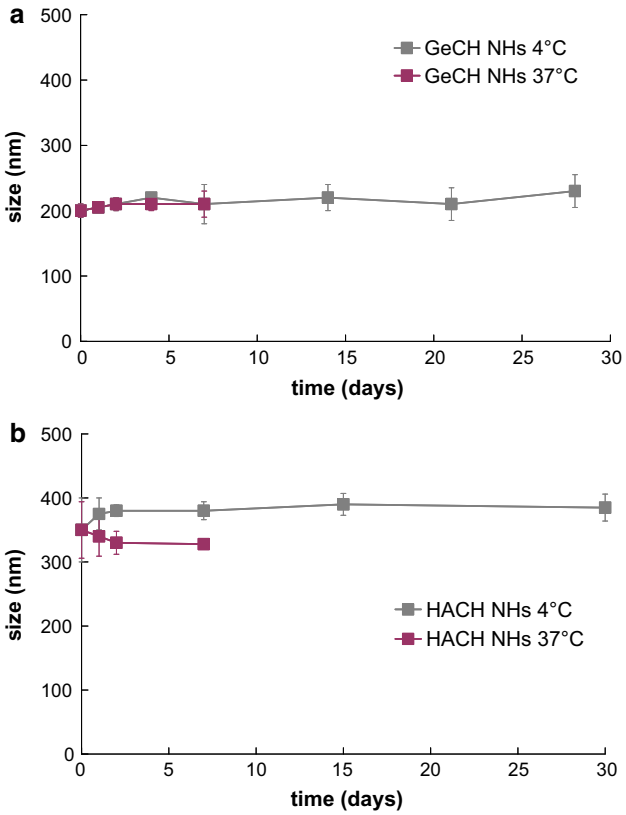

Fig. 1 Stability at 4 and 37 °C of GeCH NHs (**a**) and HACH NHs (**b**) obtained by the autoclaving process

paper [7], a similar NH prepared with gellan and prednisolone, instead of cholesterol, was able to dramatically increase the solubility of paclitaxel (up to 1,000 times) when loaded in such aggregated structures. Here we report the preparation and the characterization of GeCH and HACH NHs loaded with a soluble model drug, levofloxacin, a synthetic broad-spectrum antibacterial agent for oral and intravenous administration. Levofloxacin was added to the polymeric suspensions before autoclaving; the sterilization process yielded the drug-loaded and sterile NHs which showed dimensions and PDI values quite similar to those of empty NHs.

In particular, as reported in Fig. 4, the LVF-loaded GeCH NHs, obtained as above described, showed only a slight increase in size (about 30 nm, $P = 0.04$), while the mean diameter and PDI variation observed for HACH NHs after the LVF entrapment seemed to be not significant ($P > 0.05$).

To evaluate the drug loading efficiency, the samples were freeze-dried and the obtained products were re-suspended in NMP in order to completely dissolve the polymers and the loaded drug. The amount of LVF was detected at $\lambda = 302$ nm and the drug encapsulation efficiency (i.e. % encapsulation, w/w) was 5.0 ± 0.3 % for both the NHs.

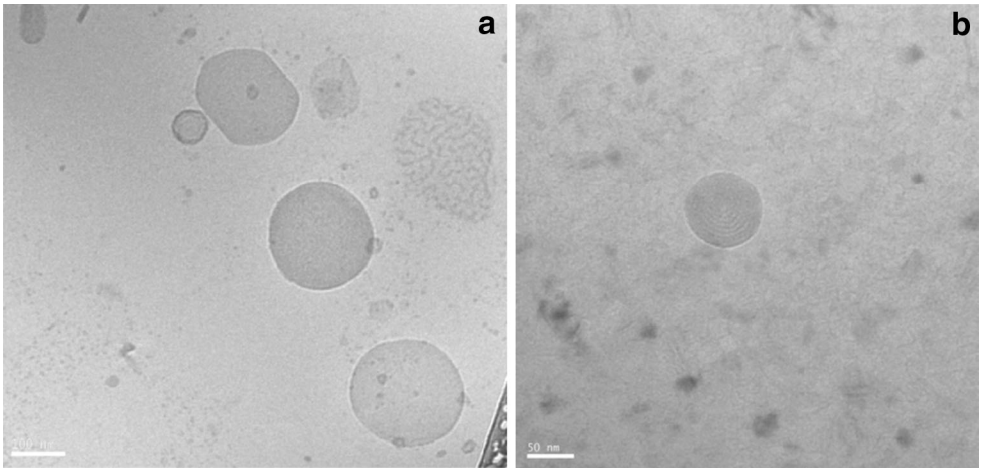

Fig. 2 Cryo-TEM micrographs of HA–CH NHs (**a**) and of GeCH NHs (**b**) obtained by the autoclaving process

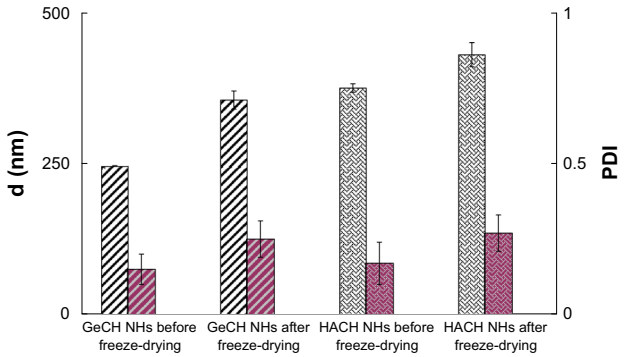

Fig. 3 Mean size (*black*) and PDI (*red*) of GeCH NHs and HACH NHs obtained by autoclaving process before and after freeze-drying procedure (Color figure online)

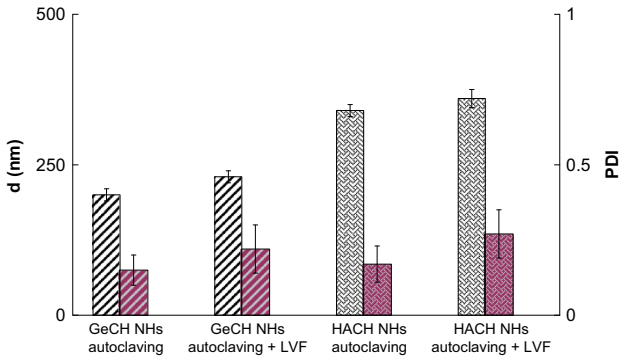

Fig. 4 Mean size (*black*) and PDI (*red*) of empty and LVF-loaded GeCH NHs and HACH NHs obtained by autoclaving process (Color figure online)

It is interesting to point out that the autoclaving procedure has been used also to sterilize pre-formed NHs and, at the same time, to improve the NH dispersion quality and uniformity. In fact, it was also possible to obtain GeCH and HACH NHs by means of bath sonication. In this case even

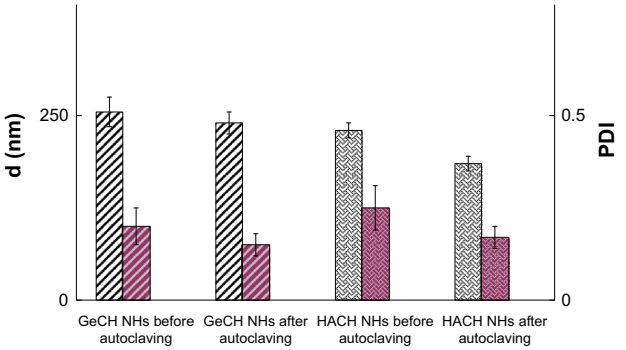

Fig. 5 Mean size (*black*) and PDI (*red*) of GeCH NHs and HACH NHs obtained by bath sonication before and after autoclaving process (Color figure online)

though the dialysis purification sometimes caused the NHs aggregation, the autoclaving process promotes the dissolution of these aggregates, thus improving the suspension features.

The GeCH NHs obtained by bath sonication showed a size of 255 ± 5 nm and a PDI of 0.15 ± 0.03. After autoclaving, GeCH NHs showed a not significant decrease in diameter, as illustrated in Fig. 5 ($P > 0.05$). Sterile suspensions of GeCH NHs were stable over 2 weeks at 4 °C (data not shown).

As illustrated in Fig. 5, the same sterilization process carried out on HACH NHs pre-formed by bath sonication induced a more significant size and PDI decrease (30 and 20 %, respectively, $P < 0.05$).

4 Conclusions

Self-assembling polysaccharide NHs are useful tools as drug carriers, which must often be sterilized before

administration. The innovative approach proposed here is based on the autoclaving of gellan-cholesterol and hyaluronic acid–cholesterol water suspensions and allows obtaining sterile gellan and hyaluronan nanoparticles, in the form of NHs, in a single step. According to this new method it is possible to avoid a separate sterilization procedure that is time consuming and, most important, in many cases is not suitable for such nanostructures, which can be remarkably affected by the sterilization conditions. Furthermore, within the same step, drugs can be loaded in the NHs, thus the obtained formulation can be directly used for administration or for vial and ampoule filling. Furthermore, in order to increase the stability of such nanoparticles, the obtained NHs dispersion can be lyophilized in the presence of a cryoprotectant and the original NHs can be re-obtained after their dispersion in water. Properties and drug loading capacity of NHs obtained by autoclaving are comparable with those of NHs prepared by sonication.

Acknowledgments Dr. Valerio Iebba and Maria Manconi are acknowledged for their valuable contribution in the data analysis. The Institut Federatif de Recherche Biologie Integrative - IFR 83, Région Ile de France, Service de Microscopie Electronique is acknowledged for CRYO-TEM analysis. Financial support from University "Sapienza" (UNIV_2013), Grant No. C26A13JTRW, is acknowledged.

References

1. Geckeler KE, Nishide H. Advanced Nanomaterials. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA publishers; 2009.
2. Gecker KE, Rosenberg E. Functional nanomaterials. Valencia: American Scientific Publishers; 2006.
3. Prasad Rao J, Geckeler KE. Polymer nanoparticles: preparation techniques and size control paramenters. Prog Polym Sci. 2011;36:887–913.
4. Coviello T, Matricardi P, Mariannecci C, Alhaique F. Polysaccharide hydrogels for modified release formulations. J Control Release. 2007;119:5–24.
5. Wacker M. Nanocarriers for intravenous injection—the long hard road to the market. Int J Pharm. 2013;457:50–62.
6. Montanari E, Capece S, Di Meo C, Meringolo M, Coviello T, Agostinelli E, Matricardi P. Hyaluronic acid nanohydrogels as a useful tool for BSAO immobilization in the treatment of melanoma cancer cells. Macromol Biosci. 2013;13:1185–94.
7. D'Arrigo G, Di Meo C, Gaucci E, Chichiarelli S, Coviello T, Capitani D, Alhaique F, Matricardi P. Self-assembled gellan-based nanohydrogels as a tool for prednisolone delivery. Soft Matter. 2012;8:11557–64.
8. Choi KY, Jeon EJ, Yoon HY, Lee BS, Na JH, Min KH, Kim SY, Myung SJ, Lee S, Chen X, Kwon IC, Choi K, Jeong SY, Kim K, Park JH. Theranostic nanoparticles based on PEGylated hyaluronic acid for the diagnosis, therapy and monitoring of colon cancer. Biomaterials. 2012;33:6186–93.
9. Zhang L, Yao J, Zhou J, Wang T, Zhang Q. Glycyrrhetinic acid-graft-hyaluronic acid conjugate as a carrier for synergistic targeted delivery of antitumor drugs. Int J Pharm. 2013;441:654–64.
10. Nakai T, Hirakura T, Sakurai Y, Shimoboji T, Ishigai M, Akiyoshi K. Injectable hydrogel for sustained protein release by salt-induced association of hyaluronic acid nanogel. Macromol Biosci. 2012;12:475–83.
11. Hurtado FK, Nogueira, Bortolini F, Silva LM, Zimmermann E, Souza MJ, Janine DM, Rolimet CMB. Determination of levofloxacin in a pharmaceutical injectable formulation by using HPLC and UV spectrophotometric methods. J Liq Chromatogr Relat Technol. 2007;30:1981–9.