Short Communication:

The effect of prolonged storage at different temperatures on the particle size distribution of tripolyphosphate (TPP) – chitosan nanoparticles

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Abstract

Chitosan nanoparticles prepared by ionotropic gelation with the tripolyphosphate (TPP) polyanion have been widely considered for drug delivery. The stability (shelf-life) of TPP-chitosan nanoparticles is highly relevant to its potential use as a drug delivery agent as this plays an important role in the function of the nanoparticle and will determine shelf-life. In the present study, the physical stability (in terms of particle size) of TPP-chitosan nanoparticles was measured across a range of different temperature conditions: 4 ºC, 25 ºC and 40 ºC using differential sedimentation. After 12 months storage at 4 and 25 °C the size of nanoparticles remained similar to those of the freshly prepared samples, whilst after storage at 40 °C there were little or no TPP-chitosan nanoparticles remaining after *only* 6 months. This may be due to the decrease in molar mass of the chitosan possibly due to hydrolysis causing scission of the polymer chains, which results in a decrease in nanoparticle size and eventual disintegration. This mechanism is important in the application of TPP-chitosan as a drug delivery agent.

Keywords: chitosan nanoparticles; tripolyphosphate (TPP); particle size; stability; degradation;

1. Introduction

Chitosan is the generic name for a family of strongly polycationic derivatives of poly-N-acetyl-D-glucosamine (chitin) extracted from the shells of crustaceans or from the mycelia of fungi (Rinaudo, 2006). In chitosan the N-acetyl group is replaced either fully or partially by NH_2 and therefore the degree of acetylation can vary from DA = 0 (fully deacteylated) to DA = 1 (fully acetylated *i.e.* chitin). Acetylated monomers (GlcNAc) and deacteylated monomers (GlcN) have been shown to be randomly distributed (Vårum, Anthonsen, Grasdalen, & Smidsrød, 1991a, 1991b).

As the only known naturally occurring polycationic polysaccharide, chitosan, and its derivatives have received a great deal of attention from, for example, the food, cosmetic and pharmaceutical industries. Important applications include water and waste treatment, antitumor, antibacterial and anticoagulant properties (Illum, 1998; Muzzarelli, 2009; Rinaudo, 2006). The interaction of chitosan with mucus is also important in oral and nasal drug delivery (Davis, & Illum, 2000; Dyer, Hinchcliffe, Watts, Castile, Jabbal-Gill, Nankervis, Smith, & Illum, 2002; Harding, Davis, Deacon, & Fiebrig, 1999). Chitosan has also been reported to enhance drug delivery across mucosal surfaces through the rearrangement of tight junction zones (Illum, 1998).

Chitosan has been widely studied in the preparation of nanoparticles for drug delivery (Anitha, Deepa, Chennazhi, Tamura, & Jayakumar, 2011; Dyer et al., 2002; Fernández-Urrasuno, Calvo, Rumuñán-Lopez, Vila-Jato, & Alonso, 1999; Gan, & Wang, 2007; Gan, Wang, Cochrane, & McCarron, 2005; Luangtana-anan, Opanasopit, Ngawhirunpat, Nunthanid, Sriamornsak, Limmatvapirat, & Lim, 2005; Morris, Kök, Harding & Adams, 2010; Shu, & Zhu, 2000; Tsai, Bai, & Chen, 2008; Xu, & Du 2005). Nanoparticles can be prepared by the electrostatic interaction and resultant ionotropic gelation between chitosan and the tripolyphosphate (TPP) polyanion (Dyer et al., 2002; Luangtana-anan et al., 2005; Janes, Calvo, & Alonso, 2001; Shu, & Zhu, 2000; Tsai, Chen, Bai, & Chen, 2011). This interaction requires only mild conditions in terms of temperature and pH (Zhang, Oh, Allen, & Kumacheva, 2004) and the nanoparticle size can be controlled by varying the chitosan:

TPP ratio, pH and the molar mass of the chitosan (Hu, Pan., Sun, Hou, Ye, Hu, & Zeng, 2008; Luangtana-anan et al., 2005; Tsai et al., 2008), although a chitosan: TPP ratio of 6:1 is considered optimal (Dyer et al., 2002; Janes, et al., 2001). Due to their sub-micron size, TPP-chitosan nanoparticles are reported to be able to penetrate into tissues via the capillaries (Gan et al., 2005).

The stability (shelf-life) of TPP-chitosan nanoparticles, in terms of particle size, is relevant to its potential use as a drug delivery agent as this plays an important role in the function of the nanoparticle (Berkland, King, Cox, Kim, & Pack, 2002; Hu, et al., 2008; López-León, Carvalho, Seijo, Ortega-Vinuesa, & Bastos-González, 2005; Luangtana-anan et al., 2005; Tang, Huang, & Lim, 2003; Tsai et al., 2008; Tsai, et al., 2011). Therefore it is fundamentally important to have the means available with which to measure the effects of and understand the relationships between storage conditions and stability.

In this paper we will look at the stability (in terms of particle size) of TPP-chitosan nanoparticles across a range of different storage temperatures: 4 ºC, 25 ºC and 40 ºC.

2. Materials and Methods

2.1 TPP-chitosan nanoparticle preparation

Chitosans (G213) from three batches (FP-002-06; FP-110-06 and FP-212-02) of DA ~ 20 % obtained from Pronova Biomedical (Oslo, Norway) and tripolyphosphate pentasodium (TPP) from Sigma Chemical Company (St. Louis, U.S.A.) were used without any further purification. Chitosans (2.0 mg/ml) and tripolyphosphate pentasodium (0.84 mg/ml) were prepared in 100 ml and 40 ml of buffer (0.2 M pH 4.3 acetate), respectively. The resultant solutions were then mixed to give an optimum TPP: chitosan ratio of ~ 1:6 (as described in Dyer et al., 2002) and the particle size distributions of the resultant nanoparticles were measured directly (t = 0).

2.2 Stability of TPP-chitosan nanoparticles

The stability of TPP-chitosan nanoparticles was determined by measuring the particle size distribution after 12 months at 4 ºC, 25 ºC or 40 ºC.

2.3 Particle size measurement

Particle size distributions were determined using a DC-18000 Disc Centrifuge (CPS Instruments, Oosterhout, The Netherlands). In order to eliminate sedimentation instability a density gradient is employed (Laidlaw, & Steinmetz, 2005). The centrifuge is accelerated to 18000 rpm and solutions of decreasing sucrose concentration (8.00 %, 7.25 %, 6.50 %, 5.75 %, 5.00 %, 4.25 %, 3.50 %, 2.75 % and 2.00 %) are injected on to the disc in 1.6 ml aliquots. After the gradient was stabilised (10 min) the TPP-chitosan nanoparticles (100 µl) were injected. Each sample was preceded by the injection (100 µl) of a PVC calibration standard (377 nm).

The time taken to sediment through a fluid of known density and viscosity to a known distance on the disc is related to particle size (Stokes, 1880). Taking into account that force varies with distance from the centre of rotor during sedimentation then Stokes' law can be expressed as follows (Laidlaw, & Steinmetz, 2005):

$$D = \sqrt{\frac{18\eta_0 \ln\left(\frac{r}{r_0}\right)}{\left((\rho - \rho_0)\omega^2 t\right)}} \qquad (1)$$

where D is the particle diameter (cm), η_0 is the viscosity of the fluid (1.15 mPas), r is the final radial displacement from the axis of rotation (cm), r_0 is the initial radial displacement (cm), ρ is the particle density (1.50 g ml^{-1}), ρ_0 is the fluid density (1.02 g ml^{-1}), ω is the rotational velocity ($2\pi rpm/60 \approx 1885$ rad s^{-1}) and t is the sedimentation time (s).

This expression results in a Stokes diameter and this equals the true diameter *only* in the case of a spherical particle; however, the correction for non-

148 sphericity is generally small (Laidlaw, & Steinmetz, 2005) and here we have
149 assumed a spherical particle based on the previous evidence (Xu & Du,
150 2005). The advantage of this method of particle size measurement is that it is
151 based on a mechanical as opposed to a mathematical separation and
152 furthermore no separation medium (other than a gradient forming material) is
153 required.
154
155 *2.4 Size Exclusion Chromatography coupled to Multi-Angle Light Scattering*
156 *(SEC-MALS)*
157 Analytical fractionation was carried out using a series of SEC columns TSK
158 G6000PW, TSK G5000PW and TSK G4000PW protected by a similarly
159 packed guard column (Tosoh Bioscience, Tokyo, Japan) with on-line MALLS
160 (Dawn DSP) and refractive index (Optilab rEX) detectors (both Wyatt
161 Technology, Santa Barbara, U.S.A). The eluent (0.2 M pH 4.3 acetate buffer)
162 was pumped at 0.8 ml min^{-1} (PU-1580, Jasco Corporation, Great Dunmow,
163 U.K.) and the injected volume was 100 µl (~1.0 x 10^{-3} g ml^{-1}). Absolute
164 weight-average molar masses (M_w) were calculated using ASTRA® (Version
165 5.1.9.1) software (Wyatt Technology, Santa Barbara, U.S.A.), at a refractive
166 index increment, *dn/dc,* of 0.163 ml g^{-1} (Rinaudo, Milas, & Le Dung, 1993).
167
168 **3. Results and Discussion**
169 *3.1 Chitosan molar mass*
170 Prior to their use in the preparation of nanoparticles the molar masses of the
171 chitosan *macromolecules* were estimated using SEC-MALS. Molar masses
172 were (294000 ± 9000); (255000 ± 9000) and (322000 ± 6000) g mol^{-1} for FP-
173 002-06; FP-110-06 and FP-212-02, respectively (**Tables 1 - 4**). As the molar
174 mass of chitosan has been reported to have an influence on the size of the
175 resultant nanoparticles (Luangtana-Anan et al. 2005) an aliquot of these
176 macromolecular solutions were stored under the same conditions as the TPP-
177 chitosan nanoparticles. The changes in molar mass (**Table 1**) were
178 consistent with our previous study (Morris, Castile, Smith, Adams and
179 Harding, 2009) in that depolymerisation is more significant at 40 °C.
180

3.2 Fresh TPP-chitosan nanoparticles

When freshly prepared, the weight-average diameters of the TPP-chitosan nanoparticles were in the range 90 – 120 nm (**Tables 1 – 3** and **Figures 1 – 3)** which is in agreement with previous studies (Anitha et al., 2011; Nasti, Zaki, de Leonardis, Ungphaiboon, Sansongsak, Rimoli, & Tirelli, 2009; Gan et al., 2005; Hu et al., 2008; Xu, & Du, 2005; Zhang et al., 2004; Tsai et al., 2011). All samples had a similar turbid (milky) appearance (turbidity was only estimated visually). Considering that the three chitosan samples were from different batches some variation in molar mass and consequently in the size of the resultant nanoparticles were expected and are consistent with findings that higher molar mass chitosans produced larger nanoparticles (Luangtana-anan et al. 2005; Tang, et al., 2003; Tsai et al., 2008; Tsai et al., 2011), although these differences were not significantly different and therefore we also have taken the mean values of particle size and molar mass for each storage condition (**Table 4**). This is still an important observation as with polysaccharide preparations batch-to-batch variation appears to be an inevitable consequence of the polydispersity of the macromolecule and it is important to determine what impact this may have on physicochemical properties in order that appropriate specifications can be defined to control the quality of the final product.

3.3 Stability of TPP-chitosan nanoparticles

It is clear from **Figures 1 – 3** that there were few or no TPP-chitosan nanoparticles present after storage at 40 °C for 12 months and this is also reflected in their turbidity (**Figure 4**). The decrease in size of the nanoparticles was evident after only 1 month storage and after 6 months the nanoparticles had essentially disappeared. This appears to be due to the decrease in molar mass of the chitosan, possibly due to hydrolysis causing scission of the polymer chains (**Tables 1 - 4**) which results in a decrease in nanoparticle size and eventual disintegration. Although, if decrease in molar mass was the only factor determining particle size and integrity we may have predicted that prolonged storage (6 months or more) at all the temperatures studied would have resulted in the decrease in size and eventual

disappearance of nanoparticles and this is clearly not the case at 4°C and 25 °C. It has been shown previously that elevated temperatures (>60 °C) have been shown to result in a decrease in size of TPP-chitosan-ascorbic acid nanoparticles (Jang, & Lee, 2008).

At 4 and 25 °C the size (110 ± 40 nm) of nanoparticles remained similar to those of the freshly prepared samples. The results at 25 °C are consistent with the findings reported in Tsai et al. (2011) for nanoparticles of a similar size range, albeit in different solvent conditions. It should however be noted that we have no information as to whether the nanoparticle *shape* has changed during storage and this may have some bearing on the apparent particle diameter.

We therefore propose that this type of nanoparticle the major cause of instability is the disintegration of the polymeric network (at higher temperatures), although we do not exclude any other mechanisms which may contribute to instability.

This mechanism is important in the application of TPP-chitosan as a drug delivery agent as drug release is reported to occur in three stages – desorption of the drug molecules from the surface; diffusion of the drug molecules through pores in the nanoparticle and degradation of the polymer network (Gan, & Wang, 2007). The rates of first two are related to the size of the nanoparticle. Furthermore the size of the nanoparticle plays an important physiological role in its *in vivo* interactions with biomolecules especially in the "protein corona" (Lundqvist, Stigler, Elia, Lynch, Cedervall, & Dawson, 2008).

4. Conclusions

The molar mass of chitosan is important in determining the size of resultant TPP-chitosan nanoparticles; higher molar mass chitosans produce larger nanoparticles. It is important to note that three batches of the same product (G213, Pronova Biomedical, Oslo, Norway) have been studied and therefore batch-to-batch variation should be given careful consideration in the

formulation of nanoparticle products, although in this case the different batches didn't produce nanoparticles of *significantly* different sizes.

TPP-chitosan nanoparticles are susceptible to instability via the disintegration of the polymeric network through chemical means, resulting in the total breakdown of the nanoparticle when stored for 6 months or more at 40 °C. Decreased particle size has been reported to increase the rate of drug delivery and also influences the shape of the drug release curve (Berkland et al., 2002). This suggests that in the present case drug delivery may be relatively unaffected by storage at 4 and 25 ℃, whereas we may see an acceleration of the drug delivery rate after prolonged storage at 40 ℃ and the performance of the nanoparticles as a drug delivery vehicle is likely to be significantly affected. We do of course realise that particle size in itself not enough to determine the stability of nanoparticles, however size is the most important (or one of the most important factors) in determining the stability of nanoparticles.

Stability could potentially be improved by using freeze-drying although the resulting formulation would require a cryoprotective agent (*e.g.* trehalose, mannitol, *etc*) (Abdelwahed, Degobert, Stainmesse, & Fessi, 2006; Eriksson, Hinrichs, de Jong, Somsen, & Frijlink, 2003).

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Table 1 Particle size parameters for TPP-chitosan (FP-002-06) nanoparticles on preparation (time = 0) and after storage at different temperatures (4 °C, 25 °C and 40 °C) for up to 12 months and the weight-average molar mass of chitosan under the same storage conditions

Time (months)	Temperature (°C)	Weight-average Molar mass ($gmol^{-1}$)	Size distribution	
			Weight-average diameter, d_w (nm)	Polydispersity index (d_w/d_n)
0	**-**	294000 ± 9000	91 ± 29	1.2 ± 0.1
1	**4**	304000 ± 12000	106 ± 39	1.3 ± 0.1
	25	285000 ± 9000	112 ± 42	1.4 ± 0.1
	40	308000 ± 9000	80 ± 19	1.1 ± 0.1
3	**4**	317000 ± 10000	109 ± 52	1.4 ± 0.1
	25	270000 ± 8000	115 ± 43	1.4 ± 0.1
	40	187000 ± 6000	78 ± 47	1.2 ± 0.1
6	**4**	285000 ± 6000	94 ± 31	1.3 ± 0.1
	25	213000 ± 2000	81 ± 20	1.2 ± 0.1
	40	152000 ± 2000	93 ± 70	1.3 ± 0.1
12	**4**	130000 ± 10000	111 ± 44	1.4 ± 0.1
	25	115000 ± 10000	100 ± 38	1.3 ± 0.1
	40	100000 ± 5000	104 ± 74	1.4 ± 0.1

Table 2 Particle size parameters for TPP-chitosan (FP-110-06) nanoparticles on preparation (time = 0) and after storage at different temperatures (4 °C, 25 °C and 40 °C) for up to 12 months and the weight-average molar mass of chitosan under the same storage conditions

Time (months)	Temperature (°C)	Weight-average Molar mass ($gmol^{-1}$)	Size distribution	
			Weight-average diameter, d_w (nm)	Polydispersity index (d_w/d_n)
0	**-**	255000 ± 9000	96 ± 28	1.3 ± 0.1
1	**4**	228000 ± 14000	113 ± 44	1.4 ± 0.1
	25	305000 ± 12000	120 ± 41	1.4 ± 0.1
	40	243000 ± 10000	85 ± 22	1.2 ± 0.1
3	**4**	217000 ± 7000	114 ± 50	1.4 ± 0.1
	25	317000 ± 10000	117 ± 58	1.4 ± 0.1
	40	167000 ± 7000	81 ± 36	1.2 ± 0.1
6	**4**	253000 ± 3000	94 ± 31	1.3 ± 0.1
	25	261000 ± 3000	84 ± 21	1.2 ± 0.1
	40	153000 ± 2000	5 ± 1	1.0 ± 0.1
12	**4**	130000 ± 10000	126 ± 49	1.5 ± 0.1
	25	115000 ± 10000	112 ± 48	1.4 ± 0.1
	40	100000 ± 5000	41 ± 23	7.3 ± 0.1

436 **Table 3** Particle size parameters for TPP-chitosan (FP.212.02) nanoparticles
437 on preparation (time = 0) and after storage at different temperatures (4 °C, 25
438 °C and 40 °C) for up to 12 months and the weight-average molar mass of
439 chitosan under the same storage conditions

Time (months)	Temperature (°C)	Weight-average Molar mass ($gmol^{-1}$)	Size distribution	
			Weight-average diameter, d_w (nm)	Polydispersity index (d_w/d_n)
0	***n.d.***	322000 ± 6000	117 ± 39	1.4 ± 0.1
1	**4**	247000 ± 17000	101 ± 34	1.3 ± 0.1
	25	273000 ± 11000	122 ± 40	1.4 ± 0.1
	40	206000 ± 12000	78 ± 17	1.1 ± 0.1
3	**4**	317000 ± 10000	114 ± 50	1.4 ± 0.1
	25	278000 ± 8000	117 ± 58	1.4 ± 0.1
	40	228000 ± 8000	78 ± 50	1.1 ± 0.1
6	**4**	*n.d.*	94 ± 31	1.3 ± 0.1
	25	236000 ± 2000	84 ± 21	1.2 ± 0.1
	40	164000 ± 2000	*n.d.*	*n.d.*
12	**4**	*n.d*	108 ± 38	1.4 ± 0.1
	25	115000 ± 10000	113 ± 36	1.3 ± 0.1
	40	100000 ± 5000	*n.d.*	*n.d.*

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Table 4 Mean particle size parameters for TPP-chitosan (G213) nanoparticles on preparation (time = 0) and after storage at different temperatures (4 °C, 25 °C and 40 °C) for up to 12 months and the weight-average molar mass of chitosan under the same storage conditions

Time (months)	Temperature (°C)	Weight-average Molar mass ($gmol^{-1}$)	Size distribution	
			Weight-average diameter, d_w (nm)	Polydispersity index (d_w/d_n)
0	**-**	290000 ± 30000	105 ± 36	1.3 ± 0.1
1	**4**	260000 ± 40000	107 ± 38	1.3 ± 0.1
	25	290000 ± 15000	119 ± 41	1.4 ± 0.1
	40	250000 ± 50000	81 ± 19	1.2 ± 0.1
3	**4**	280000 ± 60000	109 ± 48	1.4 ± 0.1
	25	290000 ± 20000	122 ± 50	1.4 ± 0.1
	40	195000 ± 20000	79 ± 43	1.2 ± 0.1
6	**4**	270000 ± 20000	94 ± 30	1.3 ± 0.1
	25	235000 ± 20000	81 ± 21	1.2 ± 0.1
	40	160000 ± 5000	9 ± 1	1.0 ± 0.1
12	**4**	130000 ± 10000	115 ± 46	1.4 ± 0.1
	25	115000 ± 10000	110 ± 41	1.3 ± 0.1
	40	100000 ± 5000	*n.d.*	1.6 ± 0.1

Legends to Figures

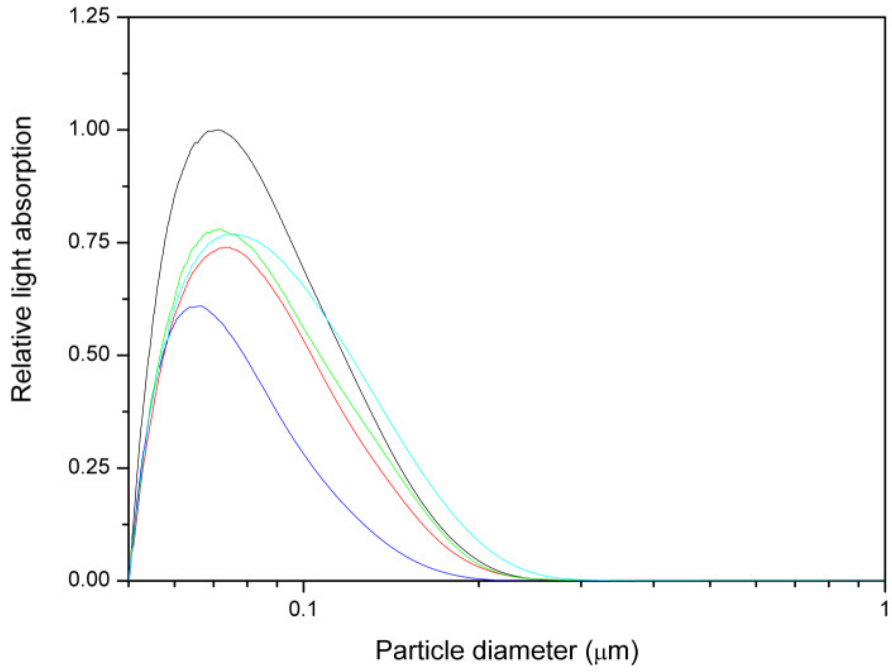

Figure 1 mean particle size distributions for TPP-chitosan nanoparticles:

— fresh nanoparticles

— stored at 4 °C for 1 month

— stored at 4 °C for 3 months

— stored at 4 °C for 6 months

— stored at 4 °C for 12 months.

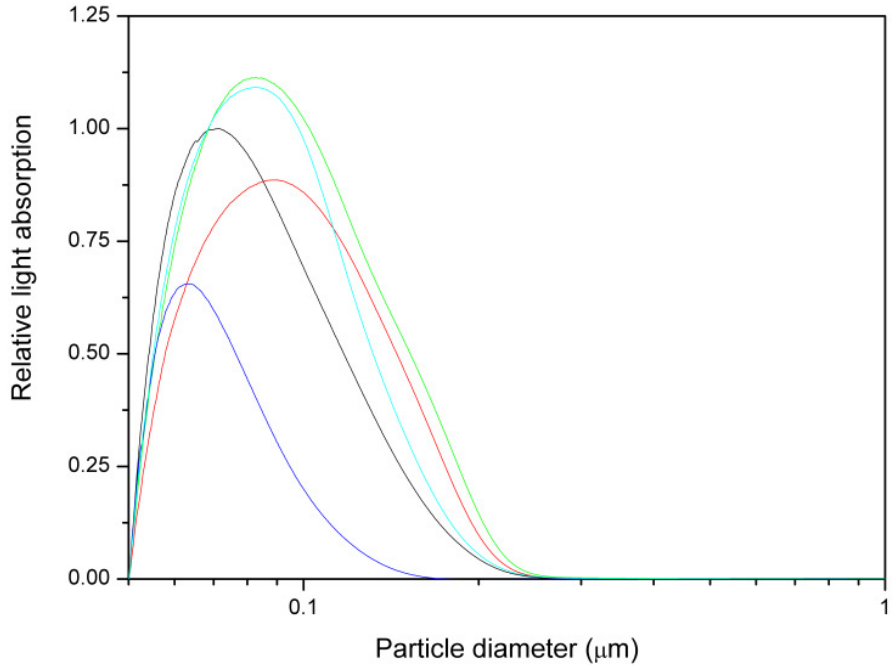

Figure 2 mean particle size distributions for TPP-chitosan nanoparticles:

— fresh nanoparticles

— stored at 25 °C for 1 month

— stored at 25 °C for 3 months

— stored at 25 °C for 6 months

— stored at 25 °C for 12 months.

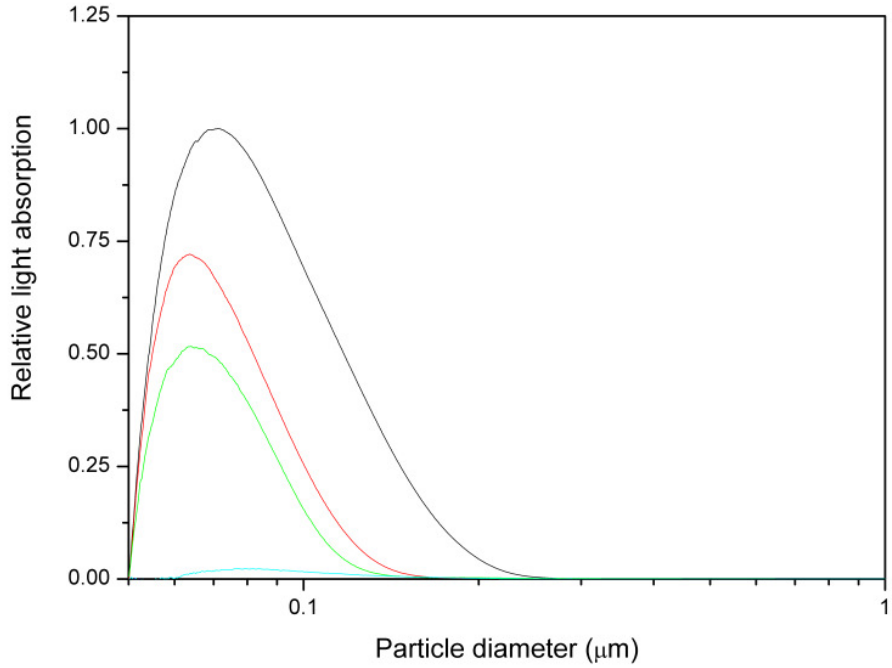

Figure 3 mean particle size distributions for TPP-chitosan nanoparticles:

— fresh nanoparticles

— stored at 40 °C for 1 month

— stored at 40 °C for 3 months

— stored at 40 °C for 6 months

— stored at 40 °C for 12 months.

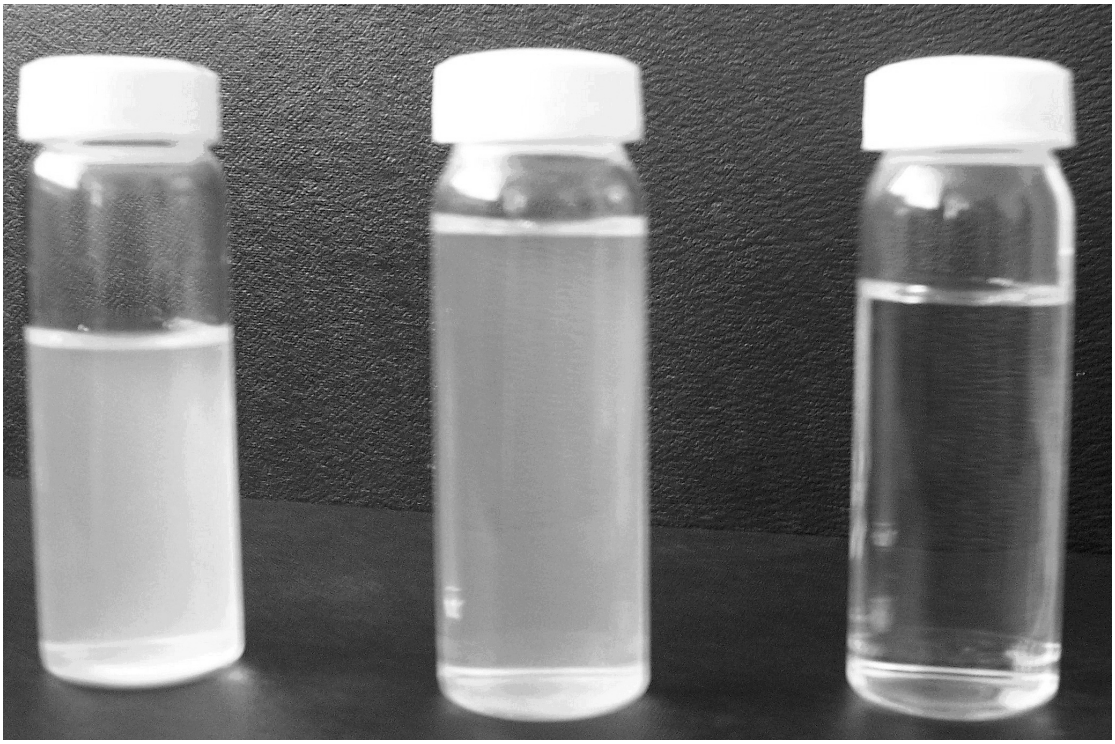

Figure 4 visual representation of the turbidity of the TPP-chitosan nanoparticle suspensions after 12 months storage at 4 °C, 25 °C and 40 °C (from left-to-right).