Supporting Information

Impact of different PEGylation patterns on the long-term bio-stability of colloidal mesoporous silica nanoparticles

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[#]These authors have contributed equally to the work.

Synthesis of the PEG-silane precursors

Poly(ethylene glycol) aminopropyl triethoxy-silane (PEG_{5000}-silane and PEG_{550}-silane). 30 g (6.0 mmol) of the commercially available poly(ethylene glycol) monomethylether (30 g for PEG with $M_w = 5000$, 3.03 mL for PEG with $M_w = 550$) in 10 mL THF were added to a solution of 864 mg (21.6 mmol) NaOH in 40 mL H_2O. The resulting mixture was stirred for 1 h at 0 °C. Then 1.37 g (7.2 mmol) *p*-toluenesulfonyl chloride in 10 mL THF was added drop wise to the reaction mixture during 1 h at 0 °C. The mixture was stirred for additional 3 h. The solution was poured onto 1 M HCl and the organic solvent was evaporated. The residue was extracted three times with chloroform and the organic phase was dried over $MgSO_4$, filtered and the solvent was removed by rotary evaporation. The transparent crude product, showing the substitution of the terminal –OH group with –OTs, was used for the next step without further purification.

It was reacted with 1.40 mL (6.0 mmol) aminopropyl triethoxysilane (APTES) in 25 mL chloroform at 70 °C for 8 h under reflux conditions, in order to bind the silane group to the PEG-OTs moiety through the amino functionality. The organic solvent was removed and the obtained raw product was stored at 4 °C and used without further purification. In the following, the PEG-silane stoichiometry was calculated based on the total content of silane in the raw product.

Synthesis of CMS nanoparticles

Unfunctionalized CMS (un-CMS). For the unmodified CMS nanoparticles, 1.92 g (9.22 mmol) of TEOS and 14.3 g (95.6 mmol) of triethanolamine (TEA) were heated to 90 °C without stirring in a

100 mL polypropylene reactor. After 20 minutes a solution of cetyltrimethylammonium chloride (CTAC, 25% in water) (2.41 mL, 1.83 mmol) in 21.7 g (1.21 mol) bi-distilled water from a Millipore system (Milli-Q Academic A10), preheated to 60 °C, was added to the polypropylene reactor. The resulting mixture, having a molar composition of 1 TEOS: 0.20 CTAC: 10.37 TEA: 130.15 H_2O, was stirred at 500 rpm at room temperature for 12 hours.

CMS-PEG$_{5000}$. The synthesis of the unmodified core of the nanoparticles was carried out as described above. 30 min after the addition of the CTAC solution to the TEOS solution, 479 mg (0.0922 mmol, 1.0 mol% of the total amount of TEOS) of the PEG_{5000}-silane, dissolved in 3 mL H_2O, together with 19.2 mg (0.0922 mmol) of TEOS were added and the synthesis mixture was stirred at 500 rpm at room temperature for 12 hours.

CMS-PEG$_{550}$. The synthesis of the unmodified core of the nanoparticles was carried out as described above. 30 min after the addition of the CTAC solution to the TEOS solution, 69.5 mg (0.0922 mmol, 1.0 mol% of the total amount of TEOS) of the PEG_{550}-silane together with 19.2 mg (0.0922 mmol) of TEOS were added and the synthesis mixture was stirred at 500 rpm at room temperature for 12 hours.

CMS-0.75PEG$_{550}$-0.25PEG$_{5000}$. The synthesis of the unmodified core of the nanoparticles was carried out as described above. After 30 min from the addition of the CTAC solution to the TEOS solution, 52 mg (0.0692 mmol, 0.75 mol% of the total amount of TEOS) of the PEG_{550}-silane and 120 mg (0.0231 mmol, 0.25 mol% of the total amount of TEOS) of the PEG_{5000}-silane, dissolved in 1 mL H_2O, together with 19.2 mg (0.0922 mmol) of TEOS was added and the synthesis mixture was stirred at 500 rpm at room temperature for 12 hours.

Template extraction. After addition of 100 mL ethanol, the CMS nanoparticles were separated by centrifugation (19000 rpm for 20 min) and redispersed in ethanol. The template extraction was performed for all samples by heating the CMS nanoparticles under reflux at 90 °C for 45 minutes in a solution containing 2 g ammonium nitrate in 100 mL ethanol, followed by 45 minutes under reflux in a solution of 10 mL concentrated hydrochloric acid in 90 mL ethanol. The CMS nanoparticles were separated from the solvent by centrifugation and washed with ethanol after each extraction step.

Characterization results

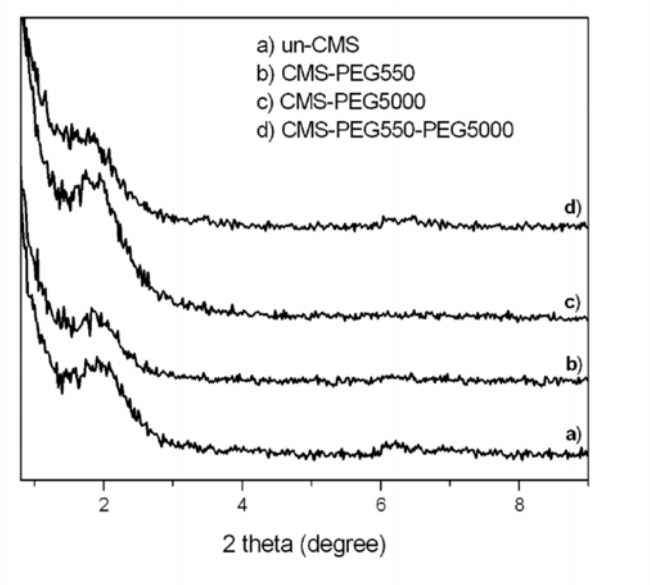

Figure S-1. Small-angle X-ray diffraction pattern to investigate the mesoporous order of the samples a) un-CMS; b) CMS-PEG550; c) CMS-PEG5000 and d) CMS-PEG550-PEG5000.

Scanning electron micrographs (Figure S-2) are also useful to determine particle size and morphology. Due to the drying process necessary for sample preparation, all samples show aggregated silica nanoparticles. However, some single nanoparticles could be detected for size measurements. The samples CMS-PEG5000 and CMS-PEG550-PEG5000 exhibit an average diameter of about 70 nm, which is around 10 nm higher in comparison to the unfunctionalized CMS nanoparticles (around 60 nm in diameter). This is tentatively attributed to the long polymer chains on these particles. Theoretically the extended PEG5000 chain should feature a length of 27 nm. However, taking into account the conformational flexibility of the polymer, lower attachment density, and contraction during drying in the SEM, the observable increase in particle diameter should be considerably smaller. The sample CMS-PEG550, consisting only of short polymer chains, has an average particle size of 64 nm, which suggests good agreement with the theoretical polymer chain length of about 3.2 nm.

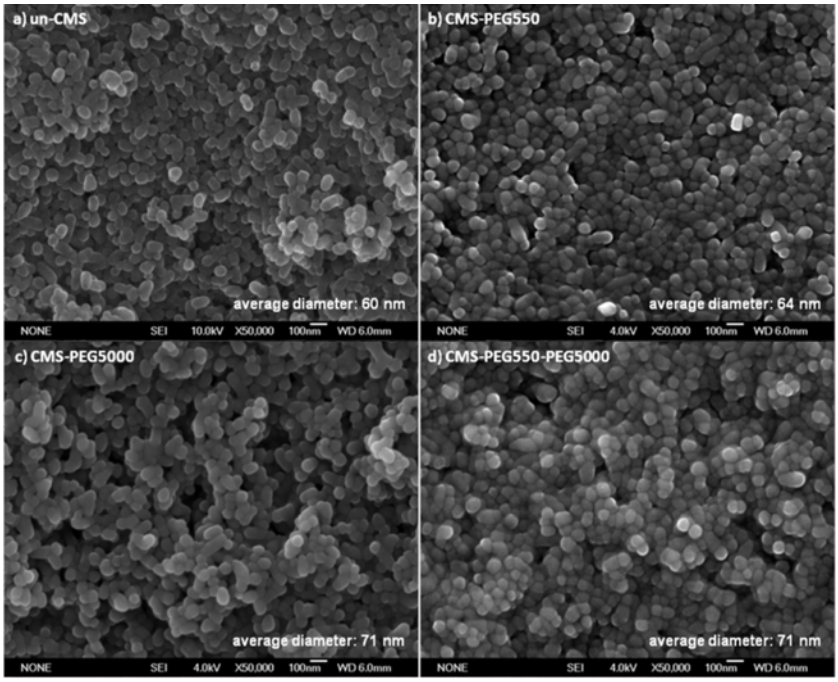

Figure S-2. Scanning electron micrographs (SEM) of (a) unfunctionalized CMS (un-CMS) and PEGylated CMS corresponding to the samples: (b) CMS-PEG550; (c) CMS-PEG5000 and (d) CMS-PEG550-PEG5000.

Zeta potential measurements were used to determine the changes of the nanoparticle surface charge. Figure S-3 indicates a shift of the isoelectric point (IEP) to much higher pH-values for the PEGylated CMS nanoparticles. The surface of the particles is positively charged almost in the whole acidic zone. These positive values are not only caused by the attached PEG-silane containing a secondary amino group, but also by the aminopropyl triethoxysilane, which is still present in the non-purified silane precursor.

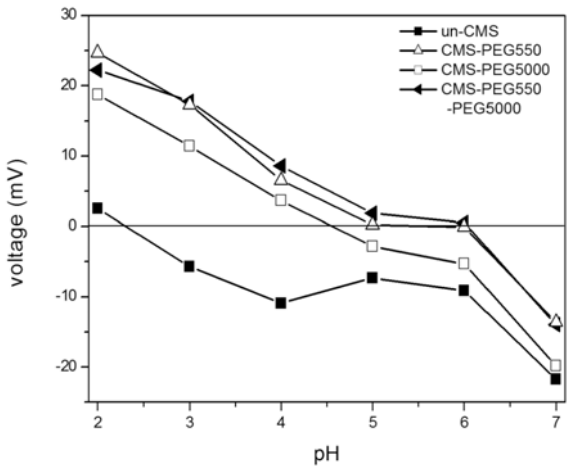

Figure S-3. Zeta potential measurements corresponding to: un-CMS (filled square); CMS-PEG550 (empty triangle); CMS-PEG5000 (empty square) and CMS-PEG550-PEG5000 (filled triangle).

^{13}C solid state NMR (Figure S-4) gives evidence of the ethylene oxide groups (peak at 70 ppm) constituting the backbone of the long PEG chains. Since the samples were extracted and extensively washed after the co-condensation procedure, no residual PEG-silane precursor should remain in the colloidal suspension of the samples. In the spectrum of unfunctionalized CMS nanoparticles (Figure S-4.a) this peak does not occur. For all samples additional peaks attributed to the ethoxy groups at 58 ppm (O-**C**H_2-CH_3) and 15 ppm (O-CH_2-**C**H_3) result from the template extraction with ethanolic solutions. The peaks related to C-N and C-Si are not visible in the spectra because of their low occurrence in the nanoparticle shell with respect to the C-O peaks of the polymer chain.

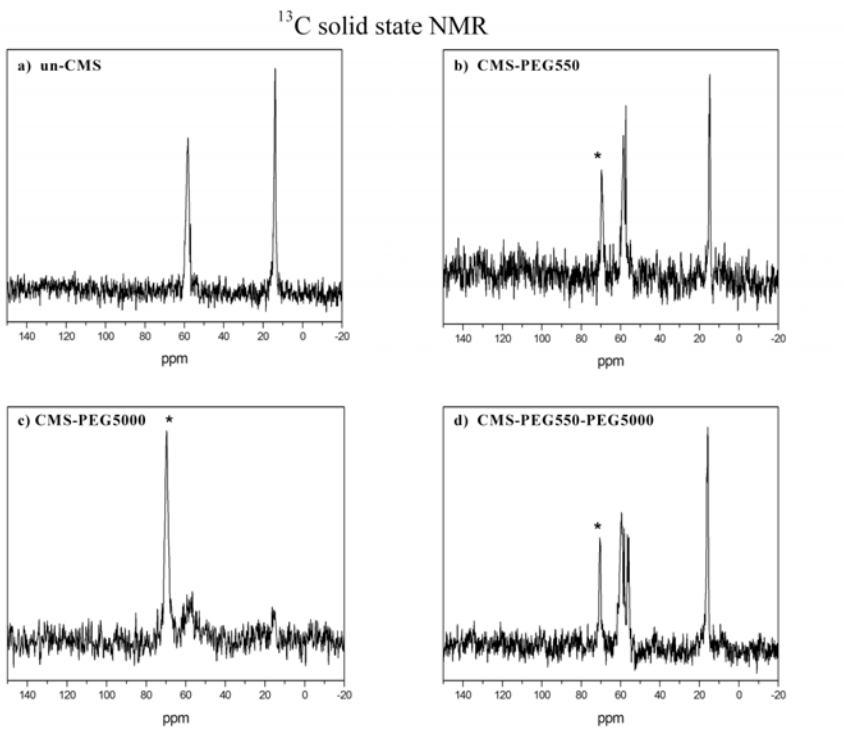

Figure S-4. ^{13}C solid state NMR data corresponding to: a) un-CMS; b) CMS-PEG550; c) CMS-PEG5000 and d) CMS-PEG550-PEG5000. The asterisk indicates the peak corresponding to the ethylene oxide groups.

By means of a mathematical model it is possible to estimate the average density of PEG at the outer surface of the mesoporous silica particles. It is assumed that the CMS nanoparticles are perfect spheres and that the mesopores are straight cylinders without defects and tortuosity. Equation S-1 shows the formula used for calculating the average number of PEG molecules per square nanometer, thus the density of PEG (D_{PEG},) at the outer nanoparticle surface.

The equation used to estimate the PEG density at the nanoparticle outer surface

$$D_{PEG}[molecule/nm^2] = \frac{\rho\beta N_A d \cdot 10^{-24}}{6M[1-\frac{\rho V_p}{3}]} \qquad \text{(S-1)}$$

Eq. S-1. Equation to calculate the density D_{PEG} of PEG chains (in molecules per nm²) at the outer surface of CMS nanoparticles: ρ *i*s the density of silica [g/cm³], β is the weight of PEG in the sample obtained by TGA data [mg/g], N_A is the Avogadro constant, d is the average particle diameter calculated from TEM images [nm], M is the molecular weight of the PEG polymer [g/mol] and V_p is the pore volume obtained by nitrogen sorption measurements [cm³/g]. The factor 10^{-24} is added to convert the m^2 into nm^2.

Since the nanoparticle is modelled as a mesoporous sphere, the silica surface offered for PEG anchoring is calculated by subtracting the void surface originating from the mesopore openings (S_{pore}) from the whole surface area of the sphere (S_{sph}). Thus:

$$D_{PEG} = \frac{m_{PEG} N_A 10^{-24}}{M(S_{sph} - S_{pore})} \qquad \text{(S-2)}$$

The mass of PEG per nanoparticle (m_{PEG}) is obtained from the mass of PEG per g of sample, calculated from TGA (β) multiplied by the weight of a nanoparticle w. Such weight w is obtained from the silica density ρ and the volume of the particle as a whole sphere V_{sph}.:

$$m_{PEG} = \beta w = \beta \rho V_{sph} = \beta \rho \frac{4}{3} \pi \left(\frac{d}{2}\right)^3 = \frac{1}{6} \beta \rho \pi d^3 \qquad \text{(S-3)}$$

The whole area of the sphere is defined as the surface area of a single nanoparticle, where the diameter is calculated from the TEM results:

$$S_{sph} = \pi d^2 \quad \text{(S-4)}$$

The void-surface due to the mesopores is defined as the base of a cylinder having as diameter (d_{pore}) the mesopore size (calculated from the DFT model from nitrogen sorption measurement) multiplied by the number of pores per nanoparticle (n_{pore}):

$$S_{pore} = n_{pore} \pi \left(\frac{d_{pore}}{2}\right)^2 \qquad \text{(S-5)}$$

Since we have assumed that mesopores are straight cylinders, completely crossing the particle volume, the number of mesopores per nanoparticle is calculated as twice the base of each cylinder, ($2n_{cylinder}$):

$$n_{pore} = 2n_{cylinder} = 2\frac{\alpha}{V_{cylinder}} = 2\frac{\alpha}{\pi d\left(\frac{d_{pore}}{2}\right)^2} \quad \text{(S-6)}$$

The number of cylinders crossing the nanoparticle is obtained by dividing the pore volume of a single nanoparticle α, expressed in m^3, by the volume $V_{cylinder}$ of a cylinder, calculated taking the nanoparticle diameter d as the height of the cylinder. α is calculated as the pore volume V_p multiplied by the mass of the nanoparticle w:

$$\alpha = V_p w = V_p \rho V_{sph} = V_p \rho \frac{4}{3}\pi\left(\frac{d}{2}\right)^3 = V_p \rho \pi \frac{d^3}{6} \quad \text{(S-7)}$$

After inserting each term in equation (S-5), the void-surface due to the mesopores results as:

$$S_{pore} = n_{pore}\pi\left(\frac{d_{pore}}{2}\right)^2 = 2\frac{\alpha}{\pi d\left(\frac{d_{pore}}{2}\right)^2}\pi\left(\frac{d_{pore}}{2}\right)^2 = 2\frac{V_p \rho \pi \frac{d^3}{6}}{\pi d\left(\frac{d_{pore}}{2}\right)^2}\pi\left(\frac{d_{pore}}{2}\right)^2 = \frac{V_p \rho \pi d^2}{3} \quad \text{(S-5)}$$

Therefore the density of PEG D_{PEG} on the outer nanoparticle surface from equation (S-2) is:

$$D_{PEG} = \frac{m_{PEG} N_A 10^{-24}}{M\left[\pi d^2 - \frac{V_p \rho \pi d^2}{3}\right]} = \frac{\frac{1}{6}\beta\rho\pi d^3 N_A 10^{-24}}{M\pi d^2\left(1 - \frac{V_p\rho}{3}\right)} = \frac{\beta\rho d N_A 10^{-24}}{6M\left(1 - \frac{V_p\rho}{3}\right)} \quad \text{(S-2)}$$

By varying the silica density ρ between 1.85 and 2.20 g/cm³,[1,2] D_{PEG} results in 1.3 – 2.1 molecules/nm² for the sample CMS-PEG550, containing short PEG polymer chains. The sample with mixed PEG polymer chain lengths (CMS-PEG550-PEG5000) shows a density of 1.5 – 2.0 molecules/nm². Sample CMS-PEG5000 with long PEG polymer chains exhibits an average density of 0.5 – 0.7 molecules/nm². These findings suggest a higher density on the particle surface for short PEG polymer chains. The long PEG polymer chains apparently need more space due to steric hindrance, resulting in a lower effective surface binding and hence smaller density.

Bio-stability assays

Table S-1. pH values of the SBF solution and structural data of the samples during the biostability assays at 37 °C. DFT pore size refers to the peak value of the pore size distribution.

Sample	pH	Pore size [nm]	BET surface area [m^2/g]	Pore volume [cm^3/g]
un-CMS				
before	7.40	3.9	1440	1.08
2 h	7.30	4.0	742	0.55
1 d	7.32	4.3	504	0.30
4 d	7.46	4.4	362	0.17
7 d	7.38	4.4	411	0.16
14 d	7.36	4.9	332	0.097
28 d	7.34	4.4	296	0.22
CMS-PEG550				
before	7.40	3.9	994	0.87
2 h	7.35	3.9	709	0.52
1 d	7.35	3.8	605	0.44
4 d	7.55	3.5	516	0.33
7 d	7.44	3.4	409	0.26
14 d	7.43	3.3	319	0.19
28 d	7.51	4.4	320	0.26
CMS-PEG5000				
before	7.40	3.8	750	0.62
2 h	7.34	3.8	648	0.47
1 d	7.38	3.7	489	0.34
4 d	7.49	3.7	467	0.31
7 d	7.49	3.8	357	0.23
14 d	7.53	3.3	397	0.22
28 d	7.57	3.5	419	0.27
CMS-PEG550-PEG5000				
before	7.40	4.0	781	0.66
2 h	7.36	3.8	621	0.43
1 d	7.37	3.8	540	0.37
4 d	7.45	3.5	489	0.31
7 d	7.48	3.5	449	0.37
14 d	7.48	3.3	343	0.20
28 d	7.51	3.7	324	0.22

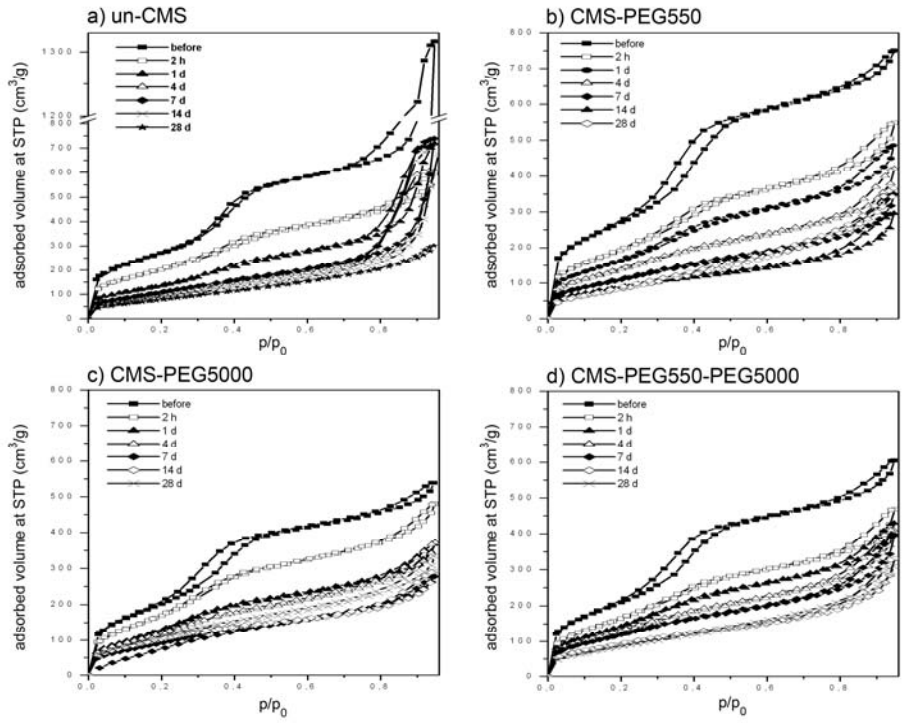

Figure S-5. Nitrogen sorption isotherms corresponding to: a) un-CMS; b) CMS-PEG550; c) CMS-PEG5000 and d) CMS-PEG550-PEG5000.

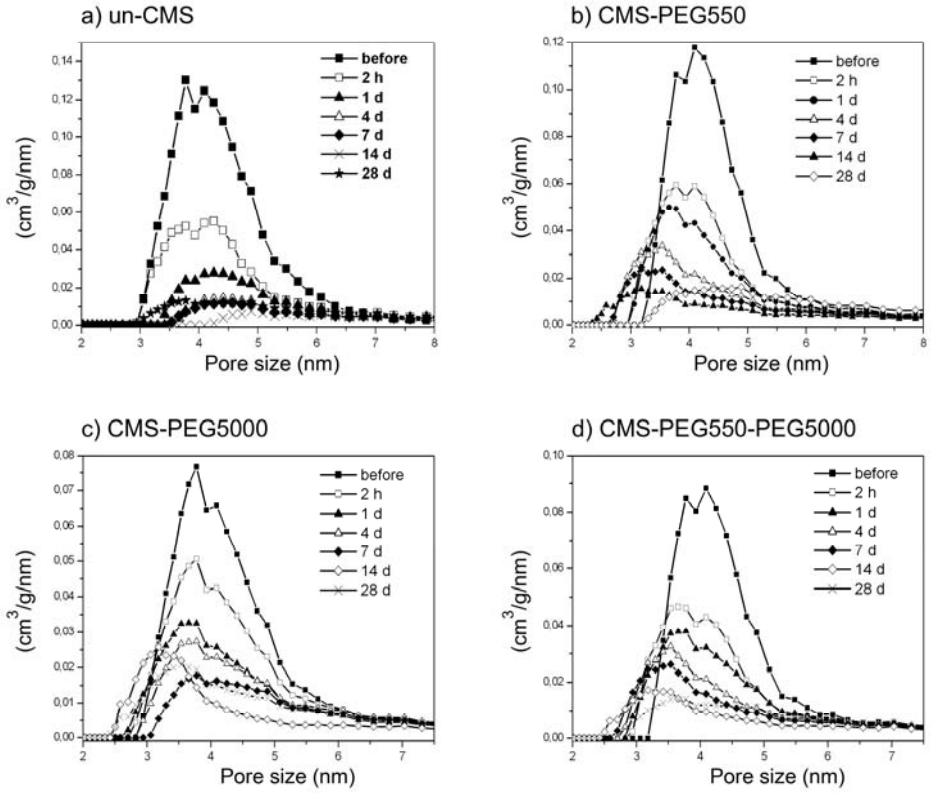

Figure S-6. DFT pore size distribution corresponding to: a) un-CMS; b) CMS-PEG550; c) CMS-PEG5000 and d) CMS-PEG550-PEG5000.

The degradation kinetics profile was fitted by a first order exponential decay equation, in order to approximately describe the decrease of the experimental BET surface area (Eq. S-8).

$$\Delta S(t) = \Delta S_0 + Ae^{-\tau \cdot t} \quad \text{(S-8)}$$

Eq. S-8: ΔS is the normalized remaining surface area in percentage of the initial value before the biostability assay as a function of time t (in days); ΔS_0 is the asymptotic value of remaining surface area to which the degraded sample tends; A is the amplitude of the decay; τ is the decay constant (in days^{-1}).

With a faster decrease of the surface area, one obtains higher values ofτ, thus the degradation exhibits a more rectangular profile. The first derivative of Eq. S-8 represents the rate of degradation calculated at the initial time t = 0 (Eq. S-9). The fitted traces are shown in Figure S-7.

$$\Delta S'(0) = -A \cdot \tau \quad \text{(S-9)}$$

Eq. S-9: ΔS'(0) is the slope of the tangent at the curve at the point t = 0.

Table S-2. Bio-degradation kinetic parameters related to the remaining BET surface area versus time after a first order exponential decay fitting.

Sample	ΔS_0	A	τ [d^{-1}]	$\Delta S'(0) = -A\tau$ [d^{-1}]	Chi^2	R^2
un-CMS	30	61.0	1.28	-78	62	0.94
CMS-PEG550	38	47.6	0.51	-24	107	0.85
CMS-PEG5000	53	34.5	0.44	-15	21	0.93
CMS-PEG550-PEG5000	45	42.7	0.25	-11	66	0.87

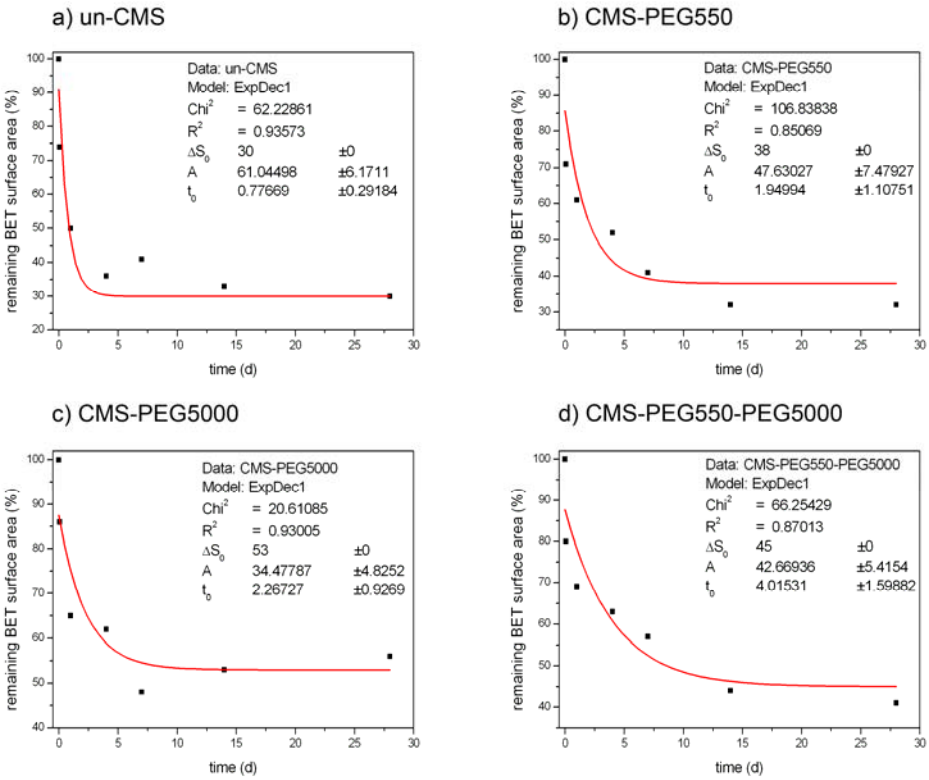

Figure S-7. Degradation kinetics profiles described by a first order exponential decay fitting for a) un-CMS; b) CMS-PEG550; c) CMS-PEG5000 and d) CMS-PEG550-PEG5000.

Small-angle X-ray diffraction (Figure S-8) shows a consistent loss of mesostructure for all samples with exposure time in SFB. The (100)-like reflections at 2° shown for all samples before the biostability assays is lost within the first days of immersion in SBF for the unfunctionalized and the CMS-PEG550 and CMS-PEG550-PEG5000 samples. Only sample CMS-PEG5000 features a persistence of this peak with decreasing intensity for up to one week of the biostability assay.

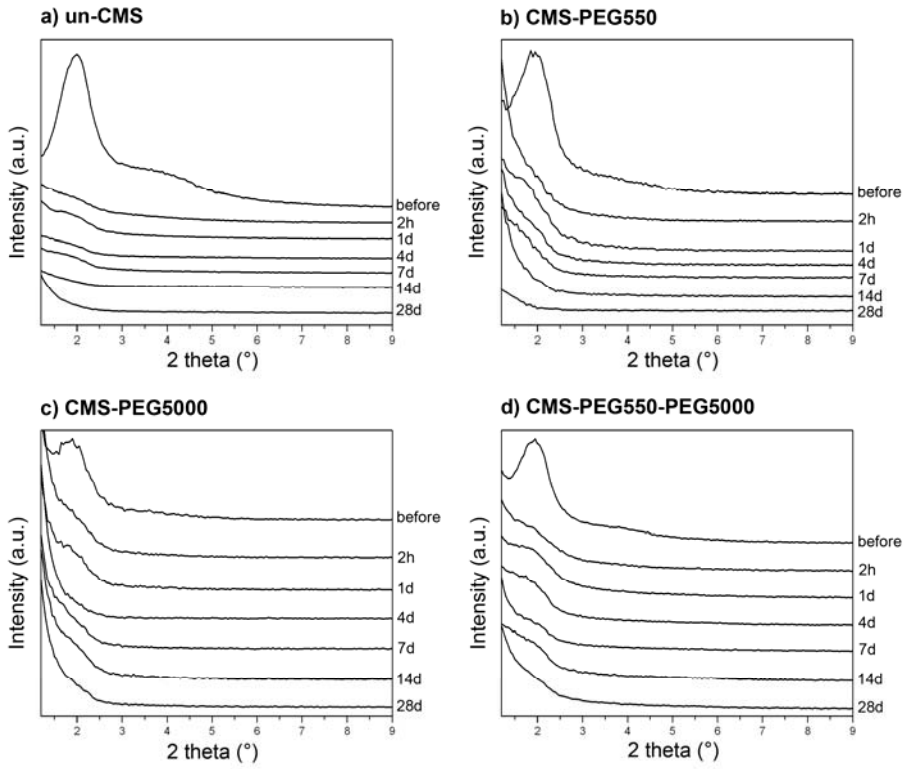

Figure S-8. Small-angle X-ray diffraction patterns as a function of exposure time of the samples a) un-CMS; b) CMS-PEG550; c) CMS-PEG5000 and d) CMS-PEG550-PEG5000 during the bio-stability assay in SBF at 37 °C.

Wide-angle XRD measurements were carried out to investigate the possible precipitation of inorganic compounds onto the CMS nanoparticle surface (Figure S-9). The reflection at 23° is attributed to the amorphous silica and the filter paper used in the sample holder for the XRD measurement. However, reflections typical for large crystalline domains of hydroxyapatite (HAp)[3] are hardly detectable both for unfunctionalized and for the PEG-coated samples. The absence of the reflections of an apatite-like layer on the particle surface indicates that the PEGylated CMS nanoparticles show no significant bioactive behavior during the time of the experiments (up to one month), although the deposition of amorphous matter cannot be excluded. For some samples a reflection at 32° is observed, which is attributed to NaCl.

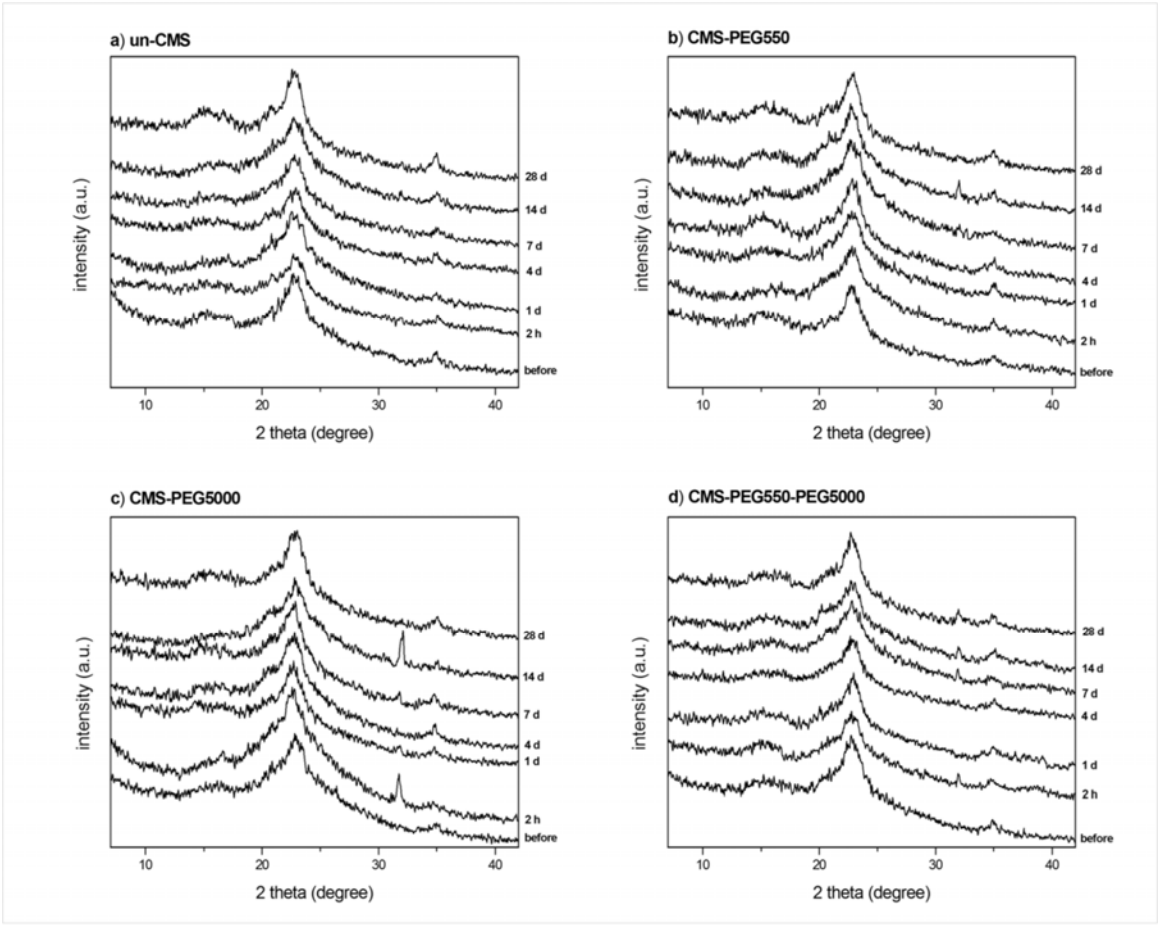

Figure S-9. Wide-angle XRD patterns as a function of exposure time of the sample a) un-CMS; b) CMS-PEG550; c) CMS-PEG5000 and d) CMS-PEG550-PEG5000 during the biostability assay in SBF at 37 °C.

Infrared spectroscopy can be used to examine the surface modification of the CMS nanoparticles, such as the possible presence of inorganic compounds (Figure S-10). After one month of biostability assay, the spectrum of the unfunctionalized CMS particles shows a slight shift of the peak from 1074 cm^{-1} to 1093 cm^{-1}, which could be tentatively attributed to the presence of phosphate (P-O stretching vibration of PO_4^{3-} tetrahedra in hydroxyl apatite).[3] Overlap of this band with the peak corresponding to the silica framework Si-O-Si asymmetric stretching mode can cause this apparent shift, which is also observed for sample CMS-PEG550. Nanoparticles coated with long polymer chains

show no shift of the peak. Furthermore a reduction of the silanol stretching mode (Si-OH at 960 cm^{-1}) occurs for all samples.

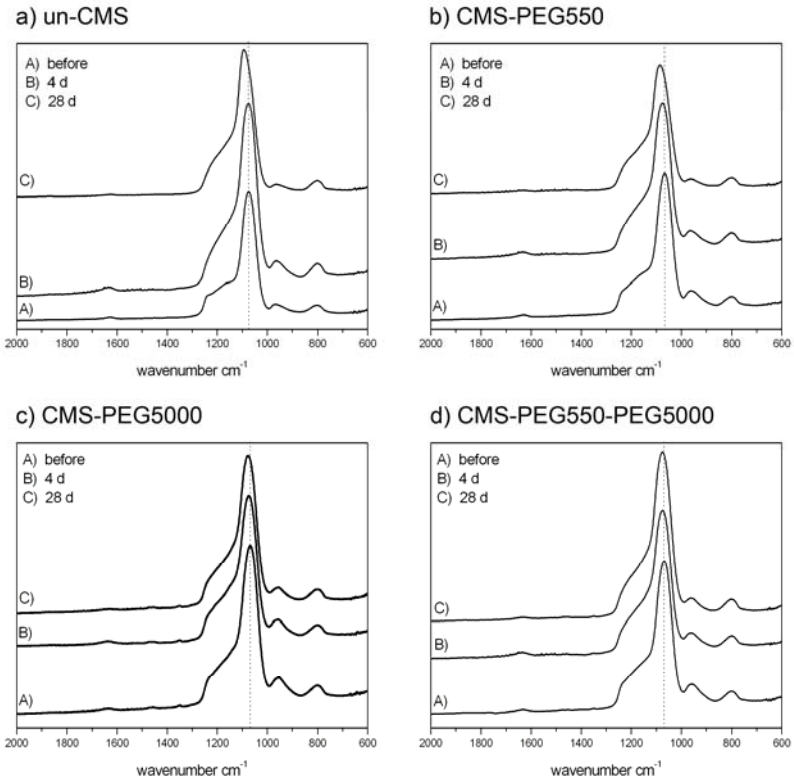

Figure S-10. Infrared spectra corresponding to: a) un-CMS; b) CMS-PEG550; c) CMS-PEG5000 and d) CMS-PEG550-PEG5000 before, after four days, and after one month of immersion in SBF at 37 °C.

The scanning electron micrograph of the un-CMS sample indicates the loss of the spherical morphology of the silica nanoparticles (Figure S-11). For all samples, precipitation of inorganic compounds occurs and EDS measurements (Figure S-12) show the presence of different elements (Na, P, S, Cl and Ca) on the nanoparticle surfaces. The molar ratios of Ca/P are calculated for the samples un-CMS: 0.60; CMS-PEG550: 1.13; CMS-PEG5000: 0.81; and CMS-PEG550-PEG5000: 1.24. Stoichometric apatite features a Ca/P ratio of 1.67; however the apatite-like layer formed on materials surfaces during in-vitro assays can consist of a calcium-deficient apatite with molar ratios of Ca/P < 1.67.[4]

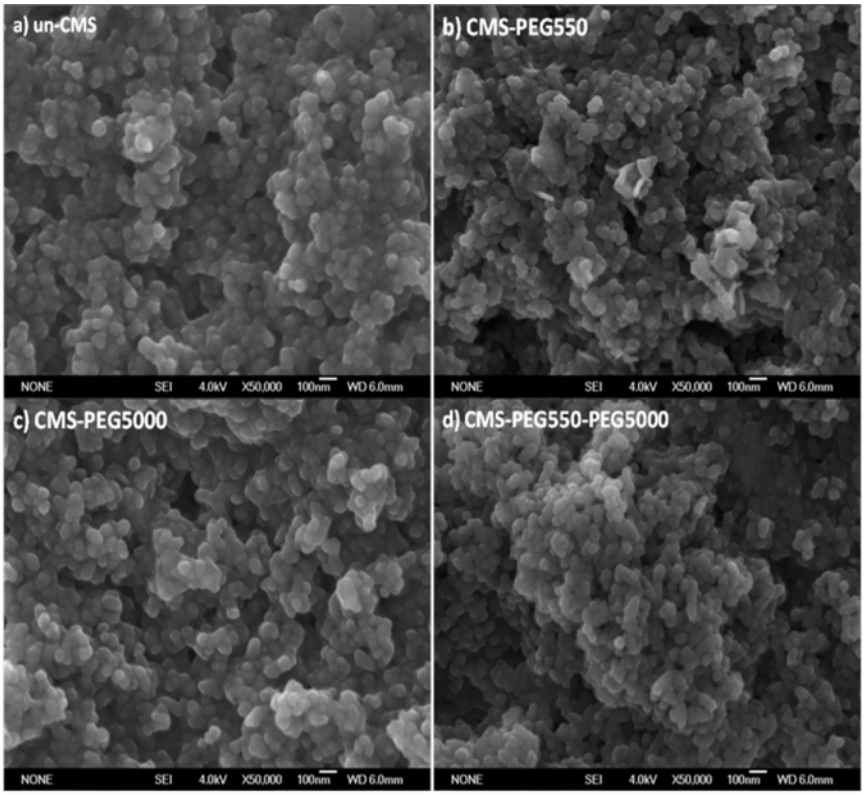

Figure S-11. Scanning electron micrographs corresponding to: a) un-CMS; b) CMS-PEG550; c) CMS-PEG5000 and d) CMS-PEG550-PEG5000, after one month in SBF solution at 37 °C.

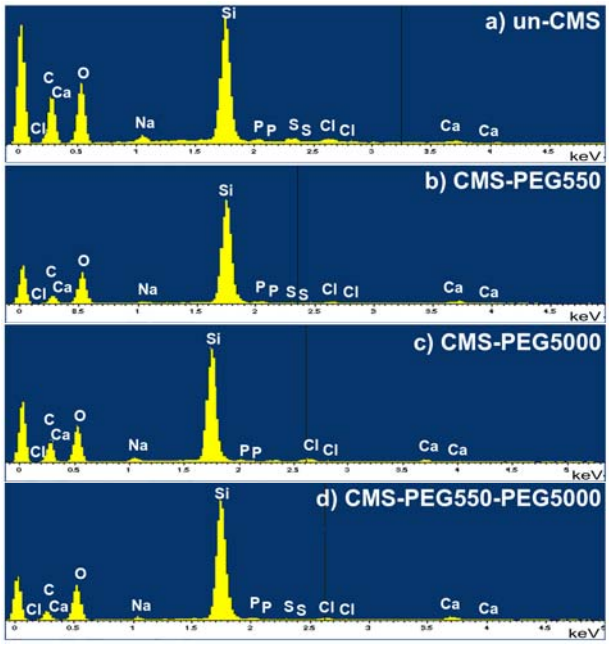

Figure S-12. Energy-dispersive X-ray spectroscopy (EDS) spectra corresponding to a) un-CMS; b) CMS-PEG550; c) CMS-PEG5000 and d) CMS-PEG550-PEG5000 after 28 days of biostability assays to detect the precipitation of inorganic compounds on the CMS nanoparticle surface.

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