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Screening poly(ethyleneglycol) micro- and nanogels for drug delivery purposes

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Abstract

This study investigates poly(ethyleneglycol) (PEG)-based micro- and nanogels, with or without lipid coating, with the aim to slowly deliver encapsulated molecules. Hydroxyethylmethacrylated PEG (PEG-HEMA), PEG-HEMA with an oligo lactate spacer (PEG-lac-HEMA), and eight-armed PEG end capped with HEMA (✳ -PEG-HEMA) were used. PEG-lac-HEMA matrices degraded very fast (in terms of days), while it took about 1 month for linear PEG-HEMA and several months for ✳ -PEG-HEMA hydrogels to become degraded. PEG-based microgels were made by use of an all aqueous technique and could be lipid-coated by mixing the microgels (made positively or negatively charged through copolymerization with respectively methacrylic acid and dimethyl aminoethyl methacrylate with a suspension of oppositely charged liposomes. The release of fluorescently labeled molecules incorporated in the PEG-based microgels could be clearly governed by the type of molecules used (lasting from hours to months). PEG-based nanogels could be made using liposomes as a nanoscopic mold, resulting in particles with a PEG gel core surrounded by a lipid coating. BSA could be easily encapsulated in the PEG nanogels which released the BSA over a period of about 1 week.

Keywords: Microgels; Nanogels; PEG; Drug delivery systems; Biodegradable; Controlled release

1. Introduction

Micrometer and nanometer sized hydrogel particles show promise for the design of advanced drug delivery devices which may enhance the therapeutic index of many types of drugs. We aim to design "simple", tiny, biodegradable hydrogel particles which, after uptake by cells, slowly release their drug load in one or another cellular compartment or in the cytosol of the cells. We aim for hydrogel particles which deliver the encapsulated drugs not only over a couple of hours, but also over days, weeks and even over months. Such slowly delivering systems may be of interest for intracellular drug delivery in long living cells such as, for example, brain cells.

Previous research from the Hennink group showed that dextran-hydroxyethylmethacrylate (dex-HEMA) has great potential for the synthesis of dex-HEMA microgels (Stenekes et al., 1998; Franssen and Hennink, 1998; Franssen et al., 1999a, b; Van Tomme et al., 2005a, b). Our group recently showed that also dex-HEMA *nanogels* can be prepared using liposomes as a nanoscopic reactor. Hereby liposomes are filled with a dex-HEMA solution which is subsequently crosslinked with the formation of (lipid-coated) dex-HEMA nanogels (Van Thienen et al., 2005, submitted). Dex-HEMA micro- and nanogels are biocompatible (De Groot et al., 2001) and degrade spontaneously under physiological conditions (van Dijk Wolthuis et al., 1997b). The degradation rate of the dex-HEMA micro- and nanogels depends on their crosslink density which is determined by the number of HEMA groups substituted on the dextran backbone (van Dijk Wolthuis et al., 1997b).

In this paper, the focus is on biodegradable poly(ethylene glycol) (PEG) micro- and nanogels. PEG has been widely studied in pharmaceutical research for various purposes as (a) it is biocompatible (Merrill and Salzman, 1982; Working et al., 1997), (b) low molecular weight PEGs are excreted by the body (Suggs et al., 1999) and (c) PEG does not severely interact with blood- and cellular proteins (Bryant and

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Anseth, 2002). Biodegradable PEG/poly(lactic acid) (PLA) and PEG/poly(glycolic acid) (PGA) block co-polymers have been investigated. When such co-polymers are end capped with acrylates they can be polymerized by UV or visible light. These macromers were polymerized in direct contact with tissues for the delivery of pDNA (Quick and Anseth, 2002), growth factors (West and Hubbel, 2002), and vaccines (Tyagi et al., 2004). Acrylate derivatized PLA–PEG–PLA is also under investigation for the production of articular cartilage: after injecting acrylated PEG solutions together with chondrocytes or growth factors, transdermal photopolymerization leads to local formation of artificial cartilage (Anseth et al., 1999; Burdick et al., 2002). Also PEG-diacrylates (without PLA or PGA blocks) (Anseth et al., 2002) and polycaprolactone-*b*-poly(ethylene glycol)-*b*-polycaprolactone (PEG-CAP) (Rice et al., 2006) are developed for the *in situ* formation of articular cartilage. PEG hydrogels can also be formed without photopolymerization: in combination with glycolide and/or lactide oligomers PEG forms a hydrogel upon injection in a 37 °C environment. Such PEG hydrogels have been reported to be non-cytotoxic (Li et al., 2003) and biodegradable under physiological conditions. They are under investigation for the controlled delivery of insuline (Kim et al., 2001; Choi et al., 2004), pDNA (Lee et al., 2003) and other hydrophilic or lipophilic drugs (for example ketoprofen (Jeong et al., 2000), spironolactone (Jeong et al., 2000), misoprostol (Lee et al., 2003), 5-fluorouracil (Qiao et al., 2005), indomethacin (Qiao et al., 2005) and paclitaxel (Kan et al., 2005)).

PEG-based hydrogels which are responsive to various stimuli have been reported as well. Andreopoulos et al. (1996, 1998) developed photoresponsive PEG-based hydrogel membranes which can be crosslinked upon exposure to >300 nm light and photodegrade when exposed to UV light (254 nm). By the co-polymerization of poly(*N*-isopropylacrylamide), temperature responsive PEG-based nanogels (Leobandung et al., 2002), microgels (Xian-Zheng and Chih-Chang, 2004) and macroscopic matrices (Ramanan et al., 2006) have been reported

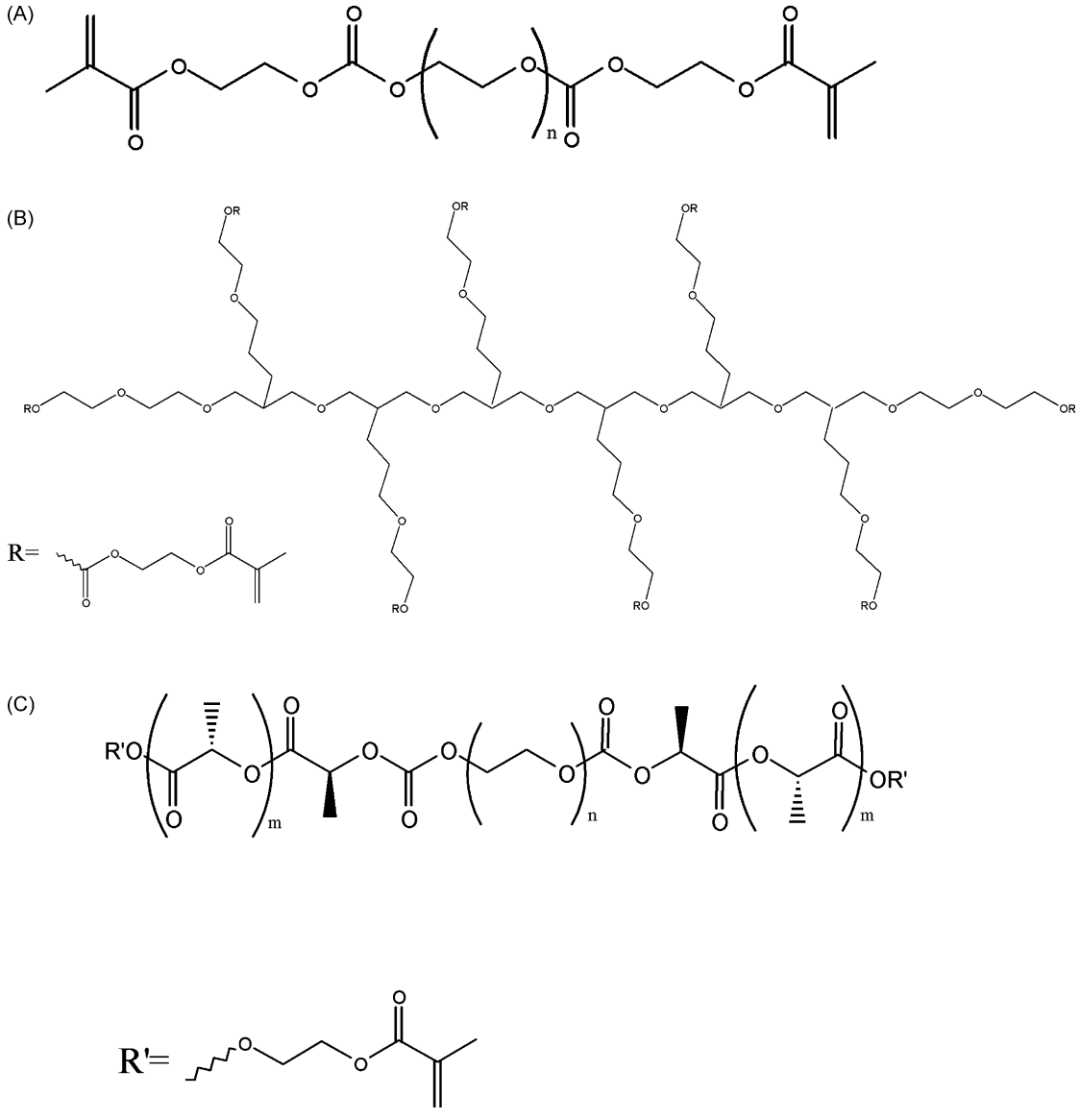

Fig. 1. Chemical structure of the monomer in PEG-HEMA (A), PEG-lac-HEMA (B) and ✳-PEG-HEMA (C).

as well. Very recently PEG-based "cell receptor responsive hydrogels" were described. Hydrogels were prepared by the interaction of multi-armed heparin modified PEG and the vascular endothelial growth factor (VEGF), a heparin binding growth factor (Yamaguchi et al., 2007). In the presence of VEGF receptors, on the surface of VEGF receptor expressing cells, VEGF is exchanged between the hydrogel and the VEGF receptor, resulting in the dissolution of the hydrogel and the release of VEGF to the cell. It was shown that such type of hydrogels increase proliferation of VEGF responsive cell lines.

The major part of previous research on PEG hydrogels concerned the synthesis, physicochemical and biological evaluation of *macroscopic* hydrogel slabs. The present study, in which PEG-HEMA (Fig. 1A), star shaped PEG-HEMA (Fig. 1B) and PEG-lac-HEMA (Fig. 1C) were used, deals with the following questions. (a) Is it possible to make PEG-based *micron-* and *nanosized* hydrogel particles? (b) Can we substantially vary the degradation time of PEG micro- and nanogels with the aim to tailor the release profiles of entrapped molecules? (c) How to surround PEG micro- and nanogels with a lipid coating as this may be of interest to modify their release characteristics and to optimize their cellular uptake and intracellular fate.

2. Materials and methods

2.1. Materials

$MgSO_4$, *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED), dimethyl aminoethyl methacrylate (DMAEMA) and hydroquinone monomethyl ether (HQM) were purchased from Fluka Chemie AG (Switzerland). 4-(*N*,*N*-dimethylamino)pyridine (DMAP) and 1,1′-carbonyldiimidazole (CDI) were from Acros Chimica (Belgium). Bovine serum albumin was purchased from Sigma–Aldrich (Germany). Lactide was purchased from Purac Biochem (The Netherlands). Poly(ethylene glycol) (PEG, M_n = 4, 10 and 20 kDa), potassium persulfate (KPS), TritonX 100 (TX 100) and dichloromethane (DCM) were purchased from Merck (Germany). Star shaped PEG (✳ -PEG, eight arms, 10 kDa) and fluorescein-isothiocyanate-PEG (FITC-PEG) were obtained from Nektar (Japan). Dextran with a molecular weight of 40 kDa (dexT40) was obtained from Amersham Bioscience (Sweden). Dialysis tubes with different molecular weight cut offs (MWCO, regenerated cellulose) were obtained from Spectrum Labs (The Netherlands). Irgacure 2959 (I2959) was a free sample from Ciba Specialty Chemicals (Belgium). SOPC, DOTAP, CHOL, DPPC, DPPG and rho-DOPE (respectively being 1-stearoyl-*sn*-glycero-3-phosphocholine;1,2-dioleolyl-3-trimethylamonium propane chloride; cholesterol, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; 1,2-dipalmitoyl-*sn*-Glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) and rhodamine-dioleolyl-phosphatidylethanolamine) were purchased from Avanti Polar Lipids (Alabaster, USA).

2.2. Synthesis of PEG-HEMA and ✳-PEG-HEMA

For the synthesis of PEG-HEMA (Fig. 1A) we refer to Van Thienen et al. (in press). The synthesis of ✳ -PEG-HEMA (Fig. 1B) is very similar to the synthesis of PEG-HEMA. In a first step, HEMA-CI is synthesized as described before (Van Thienen et al., in press). The second step, being the coupling of HEMA-CI to the terminal hydroxyl groups of PEG, occurs as follows. ✳ -PEG (M_n = 10 kDa), dried in a vacuum oven for 24 h at room temperature, and 5 mg HQM are dissolved in 10 mL DMSO in a 25 mL three-neck round bottomed flask under N_2 stream. After dissolving 0.2 g DMAP in the ✳ -PEG/DMSO solution, HEMA-CI is added drop wise while stirring vigorously. The amount of added HEMA-CI depends on how many arms of ✳ -PEG have to become substituted with HEMA and is calculated as follows (Eq. (1)):

$$m_{\text{HEMA-CI}} = \frac{m_{*\text{-PEG}} \times n}{\text{MW}_{*\text{-PEG}}} \times \text{MW}_{\text{HEMA-CI}} \times 1.5 \quad (1)$$

in which $m_{\text{HEMA-CI}}$ stands for the mass of HEMA-CI to be added, $m_{*\text{-PEG}}$ is the mass of ✳ -PEG in the solution, n is the desired number of PEG-arms to be substituted by HEMA, $\text{MW}_{*\text{-PEG}}$ and $\text{MW}_{\text{HEMA-CI}}$ are the molecular weight of respectively, ✳ -PEG (10 kDa) and HEMA-CI (224 Da). A factor 1.5 is taken into account as the incorporation efficiency of the acrylate groups on the terminal alcohol group on ✳ -PEG is not 100%.

This mixture was allowed to react for 5 days under N_2. The reaction was terminated by decreasing the pH to 4.0 with HCl. DMSO was removed by dialysis against deionized water for 1 week (MWCO of the dialysis tubes was 2 kDa), the water was refreshed twice a day. Subsequently, the solution was freeze-dried and the fluffy white powder was stored at −20 °C under N_2. Purity was determined by ^{1}H NMR in D_2O with a Gemini 300 spectrometer (Varian). The degree of substitution of the terminal alcohol groups on PEG with acrylate was determined using ^{1}H NMR from the ratio of the integral value of the acrylate (∼5.8 or 6.4 ppm) to the PEG star (∼3.6 ppm). The degree of substitution (DS; i.e. the extent of acrylation) was calculated by equation (2):

$$\text{DS} = \frac{I_{\text{CH}}}{I_{\text{CH}_2}} \times \frac{4 \times M_n}{\text{MW}_{\text{EO}}} \quad (2)$$

in which I_{CH} and I_{CH2} are the vinylic integral and the oxyethylene integral, respectively. MW_{EO} is the molecular weight of the monomer in PEG (i.e. ethylene oxide), being 44 Da. The factor 4 represents the number of protons in the ethylene oxide molecule.

Importantly, in the storage of (✳ -)PEG and in the synthesis of (✳ -)PEG-HEMA the formation of PEG peroxides should be avoided (Wade, 1999) as peroxides would spontaneously initiate the polymerization of (✳ -)PEG-HEMA solutions. To prevent peroxide formation we added HQM to the (✳ -)PEG-HEMA and stored the polymer at −20 °C under N_2.

2.3. Synthesis of PEG-lac-HEMA

The synthesis of PEG-lac-HEMA (Fig. 1C) occurred in three steps. First, L-lactide was grafted onto HEMA in the presence of stannous octoate ($SnOct_2$), yielding HEMA-lactate. Second, HEMA-lactate was activated with CDI resulting in HEMA-

lactate-CI. Third, HEMA-lactate-CI was coupled to PEG, to yield PEG-lactate-HEMA (PEG-lac-HEMA).

The synthesis of HEMA-lactate and HEMA-lactate-CI has been described in detail by van Dijk Wolthuis et al. (1997a). HEMA-lactate consisting of two lactyl residues was synthesized. The grafting of HEMA-lactate-CI to PEG occurred as follows. Fifty grams (12.5 mmol) PEG (M_n = 4 kDa), dried in a vacuum oven for 24 h at room temperature, and a spatula tip of HQM were dissolved in 500 mL DMSO in a 1 L three-neck round bottomed flask under N_2 stream. After dissolving 10 g DMAP in the PEG/DMSO solution 23 g HEMA-lactate-CI (62.5 mmol, 5 eq.) was added drop wise while stirring vigorously. This mixture was allowed to react for 4 days under N_2. The workup procedure was performed as described above for PEG-HEMA and ✳ -PEG-HEMA.

2.4. Rheological measurements on degrading PEG-based hydrogel slabs

PEG-HEMA, PEG-lac-HEMA and ✳ -PEG-HEMA solutions were polymerized by the use of KPS (0.05 g KPS/mL in PB solution) and TEMED (20% (v/v) TEMED solution in deoxygenated phosphate buffer (PB), pH was adjusted to 8.5 with HCl) following the method as described by van Dijk Wolthuis et al. (1995). Briefly, 50 μL of TEMED solution was added to 1 g of polymer solution. After homogenization, 90 μL of KPS solution was added to the system to initiate gelation.

The PEG-HEMA, PEG-lac-HEMA and ✳ -PEG-HEMA hydrogel slabs, prepared in cylindrical moulds with a diameter of 2.3 cm and a height of 2 mm, were rheologically characterized using an AR1000- N controlled stress rheometer (TA-instruments) according to a method described in detail by Meyvis et al. (1999). For G′ measurements on degrading hydrogels, the hydrogel slabs were submerged in PB at 37 °C. At regular time intervals, G′ of the hydrogel slabs was measured.

2.5. Synthesis of PEG-HEMA, ✳ -PEG-HEMA and PEG-lac-HEMA microgels

PEG-HEMA, ✳ -PEG-HEMA and PEG-lac-HEMA microgels were prepared as follows (Stenekes et al., 1998): Briefly, a deoxygenated aqueous solution of methacrylated PEG (0.25 g of a 30% (w/w) solution in PB) and a dextran solution (5 g of a 40% (w/w) solution dexT40 in PB) were mixed with each other. The mixture was vigorously mixed with a vortex for 1 min under a N_2 atmosphere to obtain a water-in-water emulsion. The resulting emulsion was allowed to stabilize for 10 min. Subsequently, 100 μL TEMED solution and 180 μL KPS solution (composition as described above) were added to the emulsion. After gentle mixing, the emulsion was incubated without stirring for 30 min at 37 °C to crosslink the methacrylate groups in the PEG chains. The crosslinked microgels were purified by three washing steps with 10 mL PB. The microgels were collected by centrifugation and resuspended in 5 mL PB.

To prepare negatively and positively charged dex-HEMA microgels respectively methacrylic acid (30 μL MAA, Fig. 2A) or dimethyl aminoethyl methacrylate (30 μL DMAEMA, Fig. 2B) were added to the starting methacrylated PEG solution. To prepare fluorescently labeled methacrylated PEG microgels, 40 μL of a FITC-PEG solution (25 mg/mL in PB) was added to the methacrylated PEG solution.

2.6. Lipid coating of (✳ -)PEG-HEMA and PEG-lac-HEMA microgels

The methacrylated PEG microgels were coated with a lipid membrane by the use of liposomes. The liposomes were prepared by dissolving 5 mg of the desired lipids (respectively, DOPC:DOTAP in a 9:1 molar ratio and DPPC:DPPG:CHOL in a 4:1:5 molar ratio) in 200 μL of chloroform. 0.05 mol% of rho-DOPE was added to make the lipid film fluorescent. The chloroform was removed by use of N_2 while spinning the test-tube gently, leaving a thin lipid film on the bottom of the tube. The remaining chloroform was removed from the lipid film by vacuum drying during for at least 4 h. The liposome dispersion was obtained by hydrating the lipid film with 1 mL PB (at a temperature at least 10 °C above the glass transition temperature during 30 min and vortexing every 5 min). For the DOPC:DOTAP liposomes, this was at 25 °C, for the DPPC:DPPG:CHOL liposomes, this was at 45 °C.

To coat the methacrylated PEG microgels, to a 1 mL (negatively or positively charged) liposome dispersion 2 mL of an (oppositely charged) microgel dispersion was added and incubated for 20 min at 25 °C to allow sorption of the liposomes to the surface of the microgels. The excess of lipid was removed by density gradient centrifugation.

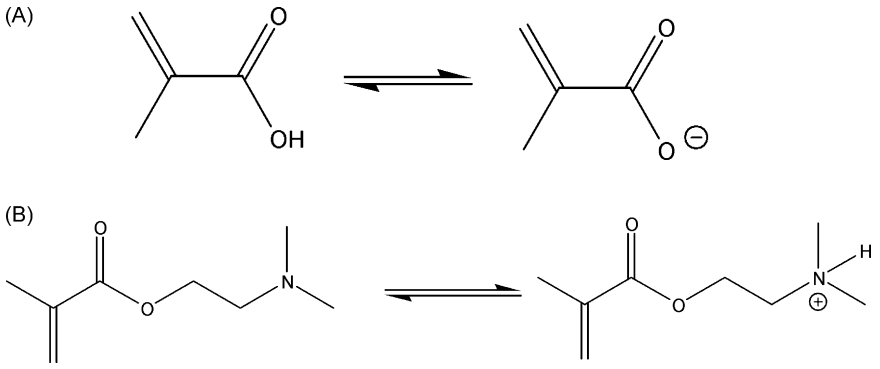

Fig. 2. Chemical structure of MAA (A) and DMAEMA (B).

2.7. Confocal laser scanning microscopy

Confocal laser scanning images were recorded with a Bio-Rad MRC 1024 microscope using a water immersion objective lens (Plan Apo 60X, NA 1.2, collar rim correction, Nikon) and a krypton/argon laser.

2.8. Release of FITC-PEG from degrading (✳-)PEG-HEMA and PEG-lac-HEMA microgels

Ten milliliters of a microgel dispersion was incubated at 37 °C and mechanically agitated. At regular time intervals the dispersion was centrifuged (3 min at 100 × *g*) and 2 mL of the supernatant was withdrawn and replaced by 2 mL of PB. The supernatant samples were stored at 4 °C until further analysis.

The fluorescence intensity of the supernatant samples was measured with a Wallac Victor 2 (Perkin-Elmer) plate reader. The fluorescence values were normalized against the fluorescence values measured at the end of the release experiments. It was verified that the measured fluorescence values belonged to the range in which a linear relation exists between the concentration of the FITC-PEG solutions and their fluorescence.

2.9. Preparation of (protein loaded) PEG-HEMA nanogels

PEG-HEMA nanogels were prepared using liposomes as a nanoscaled reactor, as previously described for dex-HEMA nanogels (Van Thienen et al., 2005). A conventional procedure to prepare the liposomes was used. A 5 mg lipid film of SOPC was made as described above. This dry lipid film was hydrated with 1 mL of a PEG-HEMA solution (i.e. 20% PEG-HEMA in 50 mM PB at pH 7.0) which contained 0.05% I2959 as a photoinitiator. The resulting dispersion was placed for 30 min at 25 °C while vortexing every 5 min. This dispersion of large vesicles was aged overnight. Next, the dispersion was extruded with a LiposoFast Pneumatic-Actuator (Avestin) provided with a 400 nm polycarbonate membrane (Whatman International). After 11 back-and-forth passages of the dispersion through the extrusion membrane, the liposome dispersion was diluted 10 times with PB. This dilution was necessary as not all the PEG-HEMA was entrapped in the liposomes. If not diluted this "free" PEG-HEMA would form a gel in the polymerization step, thereby enclosing the liposomes in a polymer matrix. The PEG-HEMA containing liposomes were subsequently exposed to UV light (365 nm from a Bluepoint 2.1 UV source, Honle UV Technology) at 25 °C during 450 s which crosslinked the PEG-HEMA solution in the liposomes with the formation of "lipid-coated PEG-HEMA nanogels". To obtain "naked PEG-HEMA nanogels" the lipid layer was removed by addition of 20.0 μL of a 100 mM solution of TritonX 100 (Merck) to 1 mL of the lipid-coated nanogel dispersion.

To prepare bovine serum albumin (BSA) loaded PEG-HEMA nanogels, BSA (50 mg/mL) was added to the PEG-HEMA solution used to hydrate the dry lipid film.

2.10. Dynamic light scattering (DLS) analysis on degrading PEG-HEMA nanogels

To study the degradation of the nanogels, a cuvette was filled with 1.2 mL of the nanogel dispersions and sealed with Parafilm™ to avoid contamination with dust particles. The cuvette was placed at 37 °C and the size distribution of the degrading particles was determined by dynamic light scattering (Autosizer 4700, Malvern Instruments) at regular times.

2.11. Protein release from degrading PEG nanogels

The amount of proteins released from the degrading PEG nanogels was measured as described by Van Thienen et al. (submitted). A Vivaspin centrifugation filtration device (having a membrane with a 300 kDa MWCO, Vivascience) was filled with a known weight (at least 2.5 g) of a dispersion of BSA loaded PEG nanogels in PB. Respectively before the nanogels began to degrade and at different times during their degradation (which occurred at 37 °C), the amount of released BSA was determined by centrifugation of the dispersion for 3 min (at 100 × *g*). The filtrate was stored (at 4 °C) for BSA analysis and PB was added to the nanogel dispersion (remaining in the Vivaspin device) until the original weight of the dispersion. The release of the BSA was measured until the nanogels were completely degraded, as could be detected by DLS measurements.

The protein concentration in the samples was measured by reversed-phase high-performance liquid chromatography (RP-HPLC, LaChrom Elite). An aliquot (2 μL) of the samples was automatically injected into a RP-HPLC system (Hitachi LaChrom Elite, L-2100 SMASH pump, L-2200 autosampler, L-2300 column oven and L-2450 Diode Array Detector). A RP C4 column (Alltech, 300 Å, 5 μm, 25 cm) was used. A water/acetonitrile mixture (in increasing ratio), adjusted to pH 2.0 with trifluoroacetic acid (TFA, Sigma) was used as the mobile phase at a constant flow rate of 0.75 mL/min. A calibration curve was obtained by injecting various volumes of a 5 mg/mL protein solution in PB. The protein concentration in the samples was calculated from the area under the BSA peak, using the calibration curve.

3. Results and discussion

3.1. Degradation behavior of PEG-HEMA, PEG-lac-HEMA en ✳-PEG-HEMA gels

Fig. 3(A–C) shows the elastic moduli of PEG-HEMA, PEG-lac-HEMA and ✳-PEG-HEMA hydrogels degrading in phosphate buffer (pH 7.0) at 37 °C. Fig. 3(A) shows that it takes approximately 6 months to degrade a PEG-HEMA (15%) hydrogel while PEG-lac-HEMA hydrogels (even those which contain 65% PEG-lac-HEMA) are already degraded after some days (Fig. 3B). It suggests that the lactate esters in PEG-lac-HEMA hydrolyze more rapidly than the carbonate esters in PEG-HEMA. This is in agreement with observations by van Dijk Wolthuis et al. (1997b) who showed that dex-HEMA degraded slower compared to dex-lac-HEMA. Fig. 3(C) shows that the

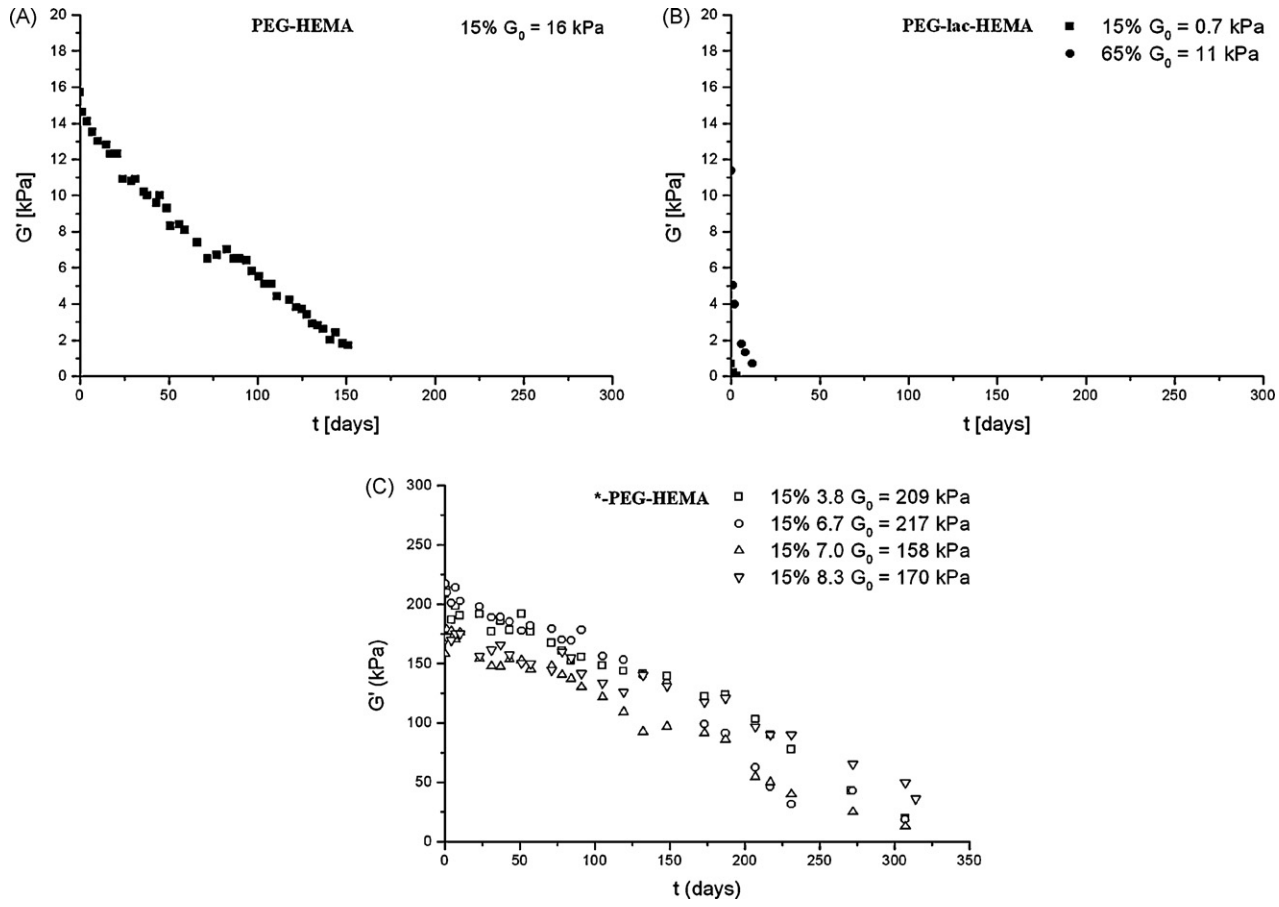

Fig. 3. Elastic moduli of degrading PEG-based hydrogels. (A) PEG-HEMA hydrogels. (B) PEG-lac-HEMA hydrogels. (C) ✳ -PEG-HEMA hydrogels. The number of arms substituted with HEMA (per ✳ -PEG) is respectively, 3.8, 6.7, 7.0 and 8.3. G_0 is the elastic modulus of the gel before degradation.

degradation of ✳ -PEG-HEMA hydrogels proceeds over several months. As ✳ -PEG-HEMA molecules have multiple methacrylate groups per molecule (note that a linear PEG-HEMA chain only bears two methacrylate groups), the concentration of crosslinks in ✳ -PEG-HEMA hydrogels is much higher than in linear PEG-HEMA hydrogels with the same PEG concentration. Subsequently, to dissolve the network more crosslinks have to be hydrolyzed which takes longer times.

Obviously, as can be seen in Fig. 3(C), increasing the DS from 3.8 till 6.7 of ✳ -PEG-HEMA hydrogels (while keeping the ✳ -PEG-HEMA concentration constant) results in a network with a higher crosslink density and thus a higher G_0 (i.e. the initial G′ value of the hydrogels before degradation). However, a further increase in DS (to 7.0 and 8.3) results in softer gels which we attribute to higher degree of intramolecular crosslinking. *Intra*molecular crosslinks – in contrast to *inter*molecular crosslinks – do not contribute to the network's elasticity (DeSmedt et al., 1997).

3.2. PEG-based microgels

3.2.1. Synthesis of PEG-HEMA, PEG-lac-HEMA and ✳ -PEG-HEMA microgels

Since aqueous solutions of PEG-HEMA and dextran are immiscible, a PEG-HEMA/dextran emulsion with dextran as continuous phase, can be made (Franssen and Hennink, 1998). When KPS and TEMED are added to PEG-HEMA/dextran emulsions, the methacrylated PEG droplets are expected to polymerize yielding PEG-based microgels. The CLSM images in Fig. 4(A_1, B_1 and C_1) show that polymerization of the emulsification indeed results in PEG-HEMA, PEG-lac-HEMA and ✳ -PEG-HEMA microgels.

To make charged PEG-microgels, we co-polymerized respectively, MAA and DMAEMA with PEG-HEMA, as this results in negatively and positively charged particles, respectively (Van Tomme et al., 2005b). Fig. 4(A_2, B_2 and C_2) shows CLSM images of respectively, PEG-HEMA-MAA, PEG-lac-HEMA-MAA and ✳ -PEG-HEMA-MAA microgels. While also positively charged PEG-HEMA-DMAEMA and ✳ -PEG-HEMA-DMAEMA could be easily produced (Fig. 4A_3 and C_3), it seemed hard to obtain PEG-lac-HEMA-DMAEMA microspheres which might be attributed to the fact that PEG-lac-HEMA-DMAEMA degrades too fast to make stable microgels (Table 1).

3.2.2. Lipid coating of PEG-based microgels

In a first approach, we tried to coat the PEG microgels by a method previously reported by Kiser et al. (1998, 2000). Therefore, (neutral) PEG microgels were sedimented on a (neutral) lipid film, however, lipid-coated PEG microgels could be hardly

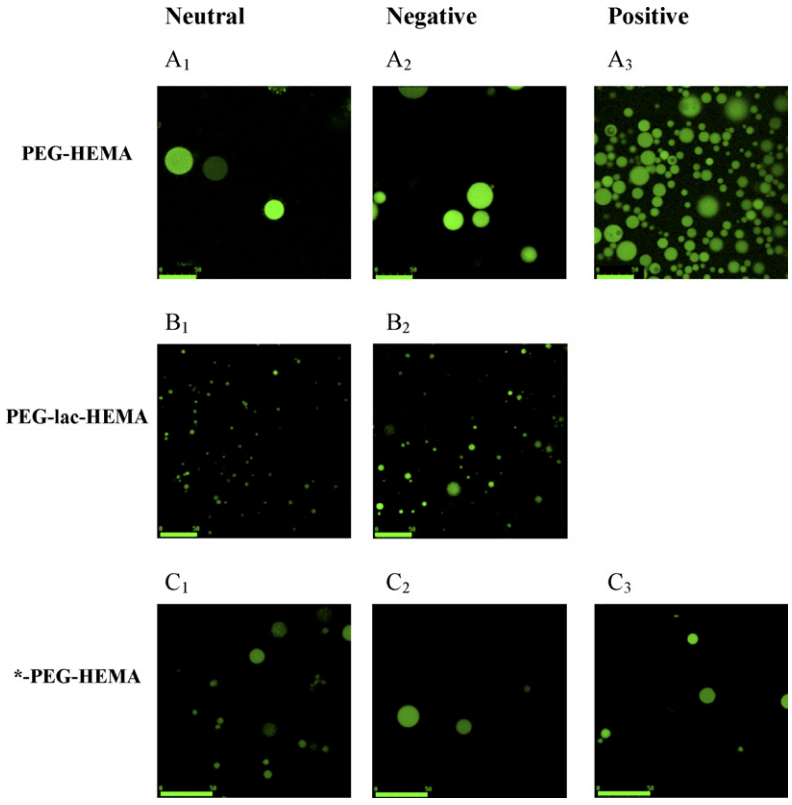

Fig. 4. CSLM images of *naked* (A) PEG-HEMA, (B) PEG-lac-HEMA and (C) ✳ -PEG-HEMA microgels. Subscript 1 refers to neutral gels, subscript 2 refers to negatively charged microgels (which contain MAA) and subscript 3 refers to positively charged microgels (which contain DMAEMA). The microgel core was fluorescently labeled with FITC-PEG.

detected by CLSM. Subsequently, following the strategy of Moya et al. (2000, 2003), positively and negatively charged PEG microgels were mixed with a suspension of oppositely charged liposomes. CLSM clearly revealed that all the PEG microgels became lipid-coated in this way (Fig. 5A–C).

As outlined in the introduction, the PEG microgels in this study are under investigation for drug delivery purposes. It might therefore be of interest to be able to optimize their surface properties. As an example, one might be interest in PEG microgels with a surface which is responsive to light (Gerasimov et al., 2005) or a change in pH (Rui et al., 1998; Boomer et al., 2003; Bergstrand et al., 2003). In that perspective, the lipid coating of the PEG microgels described above may be interesting.

3.2.3. *Release from PEG-based microgels*

Fig. 6 shows the release of FITC-PEG from degrading PEG-HEMA, PEG-lac-HEMA and ✳ -PEG-HEMA microgels. The release from both lipid-coated as well as naked (i.e. without lipid coating) microgels was investigated. The inset tabulates the time it takes to release approximately 50% of the entrapped FITC-PEG ($t_{1/2}$).

Table 1
Amount of HEMA-CI added to ✳ -PEG in the synthesis of ✳ -PEG-HEMA and the numbers of arms (out of eight) substituted with HEMA (expressed as the degree of substitution, DS), as measured by ^{1}H NMR

Molar excess HEMA-CI	DS
3	3.8
6	6.7
9	7
12	8.3

PEG-HEMA microgels deliver the encapsulated FITC-PEG over a period of approximately 1 month (Fig. 6A_1). Generally speaking, as Fig. 6(A_1) shows, the release of FITC-PEG from (neutral) PEG-HEMA, (negatively charged) PEG-HEMA-MAA and (positively charged) PEG-HEMA-DMAEMA microgels occurs similarly. Fig. 6(B_1) shows that the release of FITC-PEG from neutral and charged PEG-lac-HEMA microgels is accomplished within hours, in agreement with the findings in Fig. 3(B) which shows that PEG-lac-HEMA hydrogels degrade much faster than PEG-HEMA hydrogels. Fig. 6(C_1) shows the results of FITC-PEG release from ✳ -PEG-HEMA microgels. The release from the positively charged ✳ -PEG-HEMA microgels seems to occur much slower than from the corresponding neutral and negative ✳ -PEG-HEMA microgels.

Fig. 6(A_2, B_2 and C_2) shows the release of FITC-PEG from degrading *lipid-coated* PEG-HEMA, PEG-lac-HEMA and ✳ -PEG-HEMA microgels. The release of FITC-PEG from the lipid-coated PEG microgels seems similar to the release from the corresponding naked (i.e. non-coated) microgels (Fig. 6A_1, B_1 and C_1), indicating that the surrounding lipid membrane does not keep the FITC-PEG in the interior of the degrading PEG microgels.

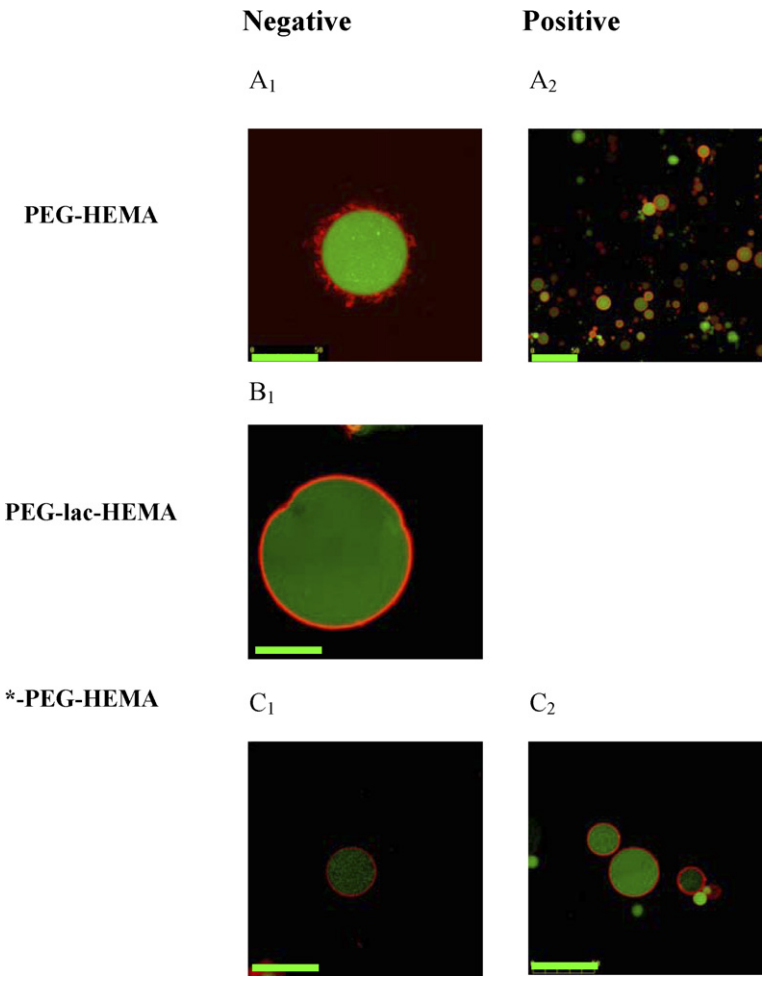

Fig. 5. CSLM images of *lipid-coated* (A) PEG-HEMA, (B) PEG-lac-HEMA and (C) ✳ -PEG-HEMA microgels. Subscript 1 refers negatively charged microgels (which contain MAA) and subscript 2 refers to positively charged microgels (which contain DMAEMA). Negative microgels were coated with DOPC:DOTAP (molar ratio 9:1) while positive microgels were coated with DPPC:DPPG:CHOL (molar ratio 4:1:5). The lipid membrane and microgel core were fluorescently labeled with respectively 5 mol% rho-DOPE and FITC-PEG.

3.3. PEG-based nanogels

3.3.1. Synthesis of PEG-HEMA nanogels

As outlined in Section 1, as we are interested in degrading gel particles which can be taken up by cells for intracellular release of encapsulated drugs, we subsequently aimed to design PEG-based nanogels, approximately, one order of magnitude smaller than the PEG microgels described above. Recently, we reported on the synthesis of biodegradable dextran nanogels, of about 400 nm in size (Van Thienen et al., 2005). We showed that lipid-coated dextran nanogels can be prepared by UV polymerization of dex-HEMA containing liposomes which were obtained by hydrating a lipid film with a dex-HEMA solution. "Naked" dextran nanogels (i.e. without lipid coating) were prepared by removing the lipid coating by TritonX 100. Using the liposome reactor technology, in this study, we aimed to obtain PEG-HEMA nanogels.

Fig. 7(A) shows the outcome of DLS measurements on lipid-coated PEG-HEMA nanogels stored in buffer (pH 8.5) at 37 °C. The PEG-HEMA nanogels were obtained by hydrating a SOPC lipid film with a PEG-HEMA solution. After extrusion of the PEG-HEMA filled liposomes, they were irradiated with UV to turn the PEG-HEMA solution into PEG-HEMA gels. After removal of the lipid coating by TritonX 100, the resulting "naked" nanogels were also stored in buffer (pH 8.5) at 37 °C. While the DLS measurements on the naked PEG-HEMA nanogels revealed that the naked nanogels get smaller in time (Fig. 7B), clearly indicating degradation, we observed that the size of the lipid-coated nanogels (Fig. 7A) did not decrease in time. One could wonder whether the PEG-HEMA gel in the lipid-coated particles did degrade. To examine this, we added TritonX 100 to the lipid-coated particles which were already in dispersion for 35 days. After removing the lipid coating, nanoparticles were no longer detected by DLS, proving that the nanogels in the interior of the liposomes were indeed degraded.

3.3.2. BSA release from degrading PEG-HEMA nanogels

We wondered whether (i) it is possible to load the PEG-HEMA nanogels with a model protein (BSA) and (ii) how the release of an encapsulated protein from PEG-HEMA nanogels looks like. Before measuring the release of BSA, we characterized the BSA loaded PEG-HEMA nanogels by DLS.

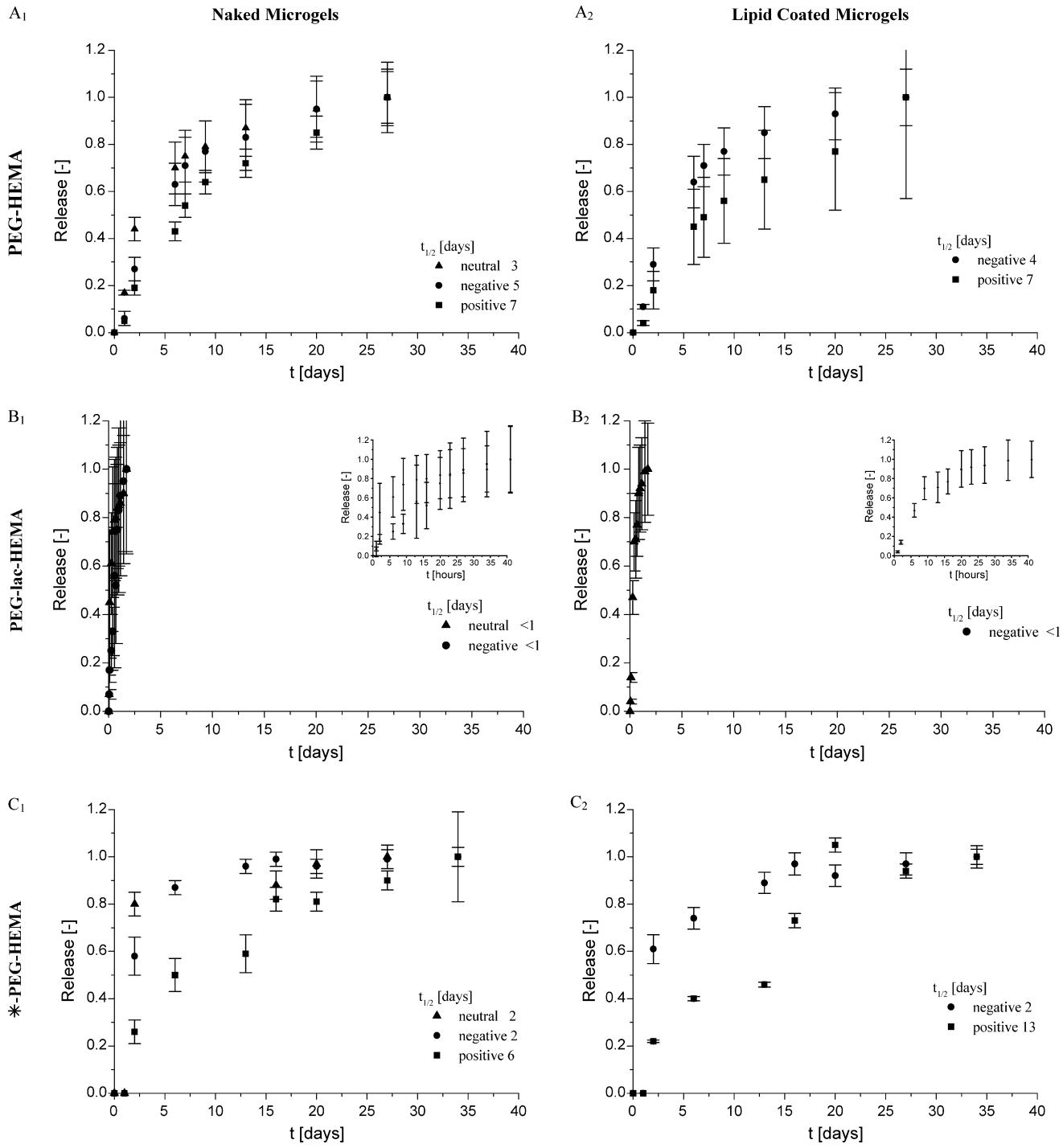

Fig. 6. Release of FITC-PEG from degrading naked and lipid-coated PEG-HEMA (A), PEG-lac-HEMA (B) and ✳ -PEG-HEMA (C, DS 3.8) microgels with (▲) neutral, (●) negative or (■) positive charge. Negative microgels were coated with DOPC:DOTAP (molar ratio 9:1) while the positive ones were coated with DPPC:DPPG:CHOL (molar ratio 4:1:5). One hundred percent release corresponds to the amount of FITC-PEG released at the end of the experiment, when the particles were completely degraded.

Fig. 8(A) shows the outcome of DLS measurements on liposomes obtained by hydrating a SOPC lipid film with a PEG-HEMA solution containing BSA. The PEG-HEMA filled liposomes are approximately 400 nm in size which is in accordance with the size of the pores of the used extrusion membrane, and show a rather narrow size distribution. Also, polymerization of the PEG-HEMA/BSA solution to obtain lipid-coated (BSA-containing) PEG-HEMA nanogels did not alter the size and polydispersity of the particles (Fig. 8B). To obtain naked PEG-HEMA/BSA nanogels the lipid coating was removed by addition of TX 100. Fig. 8(C) shows the presence of about 400 nm sized naked PEG-HEMA/BSA nanogels, besides the presence of micelles (about 12 nm in size, as formed by TX 100 and SOPC lipids).

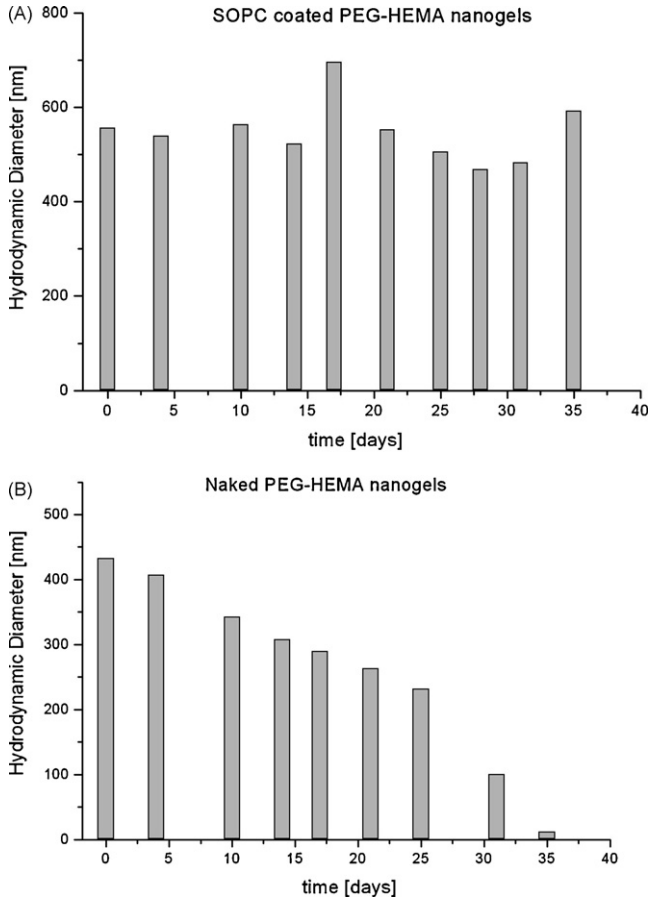

Fig. 7. Hydrodynamic diameter, as measured by DLS, of (A) SOPC-coated PEG-HEMA nanogels and (B) naked PEG-HEMA nanogels stored in PB (pH 8.5) at 37 °C. To prepare the coated PEG-HEMA nanogels the SOPC lipid film was hydrated with a 20% PEG-HEMA solution. To prepare the naked nanogels, the coated nanogels were exposed to TX 100. The shown data are the result of one set of experiments. Repeated experiments revealed the same results.

Fig. 9 shows the release of BSA from degrading PEG-HEMA nanogels. The following observations are made. First, the release of BSA starts immediately: after 3 days more than 50% of the BSA has been released. Second, after approximately 7 days BSA

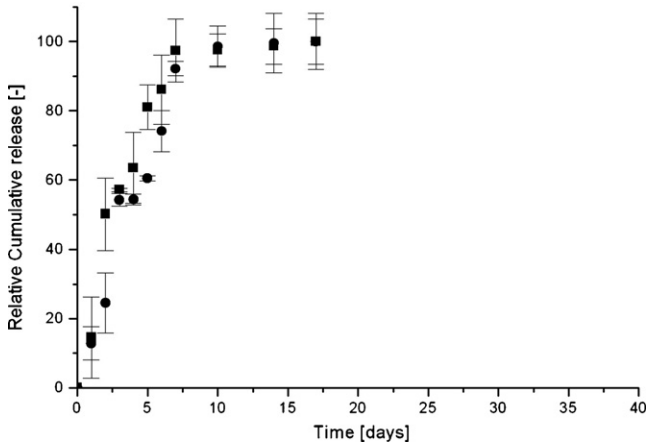

Fig. 9. Cumulative release of BSA from degrading PEG-HEMA nanogels with (■) or without (●) SOPC coating. The lipid coating was hydrated with a 20% PEG-HEMA solution. One hundred percent release corresponds to the amount of BSA released when the nanogels were completely degraded (i.e. when nanogels could no longer be detected by DLS).

release does no longer occur, although dex-HEMA nanogels could still be detected by DLS (even up to 35 days, see Fig. 7B). Release from degrading hydrogel matrices may occur diffusion-controlled and/or degradation-controlled. Highly likely, the release of BSA from the PEG-HEMA nanogels is controlled by the degradation of the PEG-HEMA matrix. Cruise et al. (1998) estimated the pore size of PEG-diacrylate hydrogels (4 kDa, 20%, in which BSA could not permeate) to be 2.2 nm. Subsequently, we assume that the average pore size of the intact (i.e. not yet degraded) PEG-HEMA matrix in the nanogels is smaller than the size of BSA molecules (i.e. 7.2 nm (Van Tomme et al., 2005a)). When degradation proceeds, the average pore size increases resulting in release of BSA. A third observation of Fig. 9 is that the release of BSA from the lipid-coated and naked PEG-HEMA nanogels occurs similarly. This is rather surprising since we observed before that BSA is not released from SOPC liposomes filled with a BSA solution (Van Thienen et al., submitted). The following consideration may be important to explain our observations. First, degradation of a PEG-HEMA

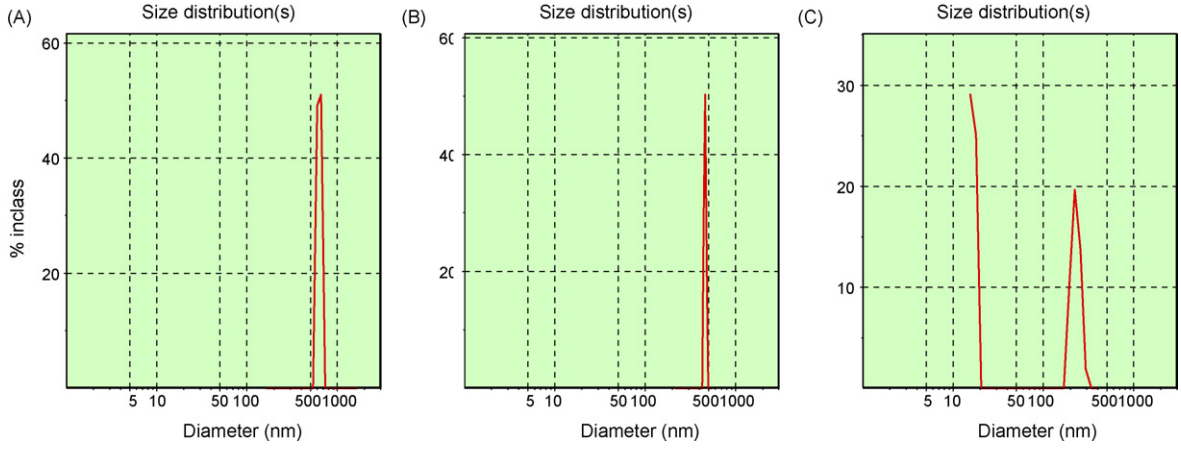

Fig. 8. Size distribution, as measured by DLS, of SOPC-coated PEG-HEMA nanogels loaded with BSA. A 20% PEG-HEMA solution was used to hydrate the SOPC lipid film. (A) The SOPC liposomes before polymerization of the PEG-HEMA, (B) the SOPC-coated PEG-HEMA nanogels after polymerization and (C) the particles after removal of the lipid coating.

gel results in a PEG solution. As we showed before, this may rise the osmotic pressure. (Van Thienen et al., in press) Subsequently, the increase in the osmotic pressure of the gel core in the lipid-coated PEG-HEMA nanogels may deform (and/or destroy) the lipid coating which allows the release of BSA into the medium.

4. Conclusions

In this study, PEG-HEMA, PEG-lac-HEMA and ✳ -PEG-HEMA hydrogels were investigated. Rheological measurements on hydrogel slabs showed that PEG-lac-HEMA matrices degrade very fast (in terms of days), while it takes about 1 month for linear PEG-HEMA and several months for ✳ -PEG-HEMA hydrogels to become degraded. We showed that, under all aqueous conditions PEG-HEMA, PEG-lac-HEMA and ✳ -PEG-HEMA microgels can be obtained by emulsion polymerization, making use of the principle of the immiscibility between dextran and PEG. Positively and negatively charged PEG microgels could be lipid coated by mixing them with oppositely charged liposomes. While the applied lipid coating did not influence the release of fluorescently labeled PEG from the microgels, the release was clearly influenced by the type of methacrylated HEMA used (lasting from hours to months). Lipid-coated PEG-HEMA nanogels were obtained by UV polymerization of a PEG-HEMA solution in the interior of liposomes. While it took about 1 month for complete degradation of the PEG-HEMA nanogels, as measured by dynamic light scattering, encapsulated BSA was completely released after 7 days. It will further be investigated how such tiny PEG hydrogel particles release encapsulated molecules after uptake by cells.

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