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Biocompatible Polyurea Dendrimers with pH-Dependent Fluorescence**

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Dendrimers are synthetic polymers with a well-defined and highly structured layered three-dimensional architecture with low polydispersity and high functionality; dendrimers can reach the size of nano-objects with dimensions similar to proteins.[1] This unique architecture and the functionality at this scale make dendrimers excellent carrier molecules for use in nanoscale medical applications.[2] Poly(amidoamine) (PAMAM)-type dendrimers are actually the most tested dendrimers as drug vectors; however their cytotoxicity is a major drawback. A few efforts have been made to overcome this problem, namely the synthesis of biodegradable dendrimers with ester linkages in their backbone.[3] Another striking feature of dendrimers is their weak blue fluorescence in aqueous solution (stronger after oxidation or acidification), a phenomenon that has been recently fully understood.[4] Therefore, since water-soluble fluorescent conjugated polymers have been described as promising tools for highly sensitive biosensing; cell imaging; and disease diagnostics,[5] dendrimers emerge as powerful nanotools.

Herein, we describe the synthesis and properties (up to the fourth generation in this report) of polyurea (PURE) dendrimers. PURE dendrimers are a new family of water-soluble blue photoluminescent biocompatible and biodegradable "green" bifunctional dendrimers. The synthesis was performed in supercritical carbon dioxide ($scCO_2$) by an economic, clean, simple, one-pot divergent-iterative approach. Our methodology is based on the synthetic strategy reported for the clean and efficient synthesis of ureas in $scCO_2$,[6] taking advantage of CO_2 as an alternative nontoxic, nonflammable, and relatively low-cost solvent.[7] Besides being a solvent for monomers and a non-solvent for polymers, CO_2 allows their easy separation at the end of a reaction by simple depressurization. The reaction of the amino groups with CO_2, which is usually viewed as an undesirable side reaction is in this case crucial for the synthesis, because we take advantage of its use not only as solvent but also as a reagent (C1 feedstock). There are only a few reports on the synthesis of hyperbranched polyureas.[8] In all cases the polymers are obtained by the reaction of amino groups and highly reactive isocyanate intermediates (obtained from protected azides) by using multi-step synthetic routes, and the polymers are not water-soluble.

In the first step, readily available tris(2-aminoethyl)amine (TREN) reacts with CO_2 under supercritical conditions in the presence of bis(trimethylsilyl)acetamide (BSA). The carbamate intermediate **1** is generated in situ by TREN silylation. After depressurization of the high-pressure cell more TREN and BSA are added to **1**, and the mixture is heated at 120 °C leading to the first-generation PURE dendrimer (**G1**). Repetitive activation/growth reaction led to PURE dendrimers of higher generations (up to generation 4) as water-soluble yellow viscous oils in quantitative yield (Scheme 1).

For the characterization of PURE dendrimers FTIR, ^{1}H NMR, and ^{13}C NMR spectra were acquired. Regarding the FTIR spectra, the most important band is the urea carbonyl stretching in the range 1638–1648 cm^{-1}, indicative of the CO_2 incorporation into the dendrimers' backbone. A band corresponding to the terminal amino groups (ca. 3340 cm^{-1}) is also present. In the ^{1}H NMR spectra, the proton signals corresponding to the methylene groups adjacent to the ureas present a chemical shift in the region $\delta = 3.0$–3.2 ppm and an increase in intensity with increasing dendrimer generation. The same behavior is observed for the signal corresponding to the amino groups (δ ca. 1.9 ppm). A typical peak broadening is also observed for higher generations. The signals at $\delta = 160$–165 ppm in the ^{13}C spectra indicate the existence of urea carbonyl groups with different microenvironments (see spectra in the Supporting Information).

The molecular weight of PURE dendrimers was hard to determine by using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The choice of the matrix was found to be critical, and only with *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) a general molecular-weight distribution was observed in linear mode with loss of signal intensity and spectral line structure (see the Supporting Information). The use of other common matrices such as 2,5-dihydroxybenzoic acid (DHB), DHB/fucose, 1,8,9-anthra-

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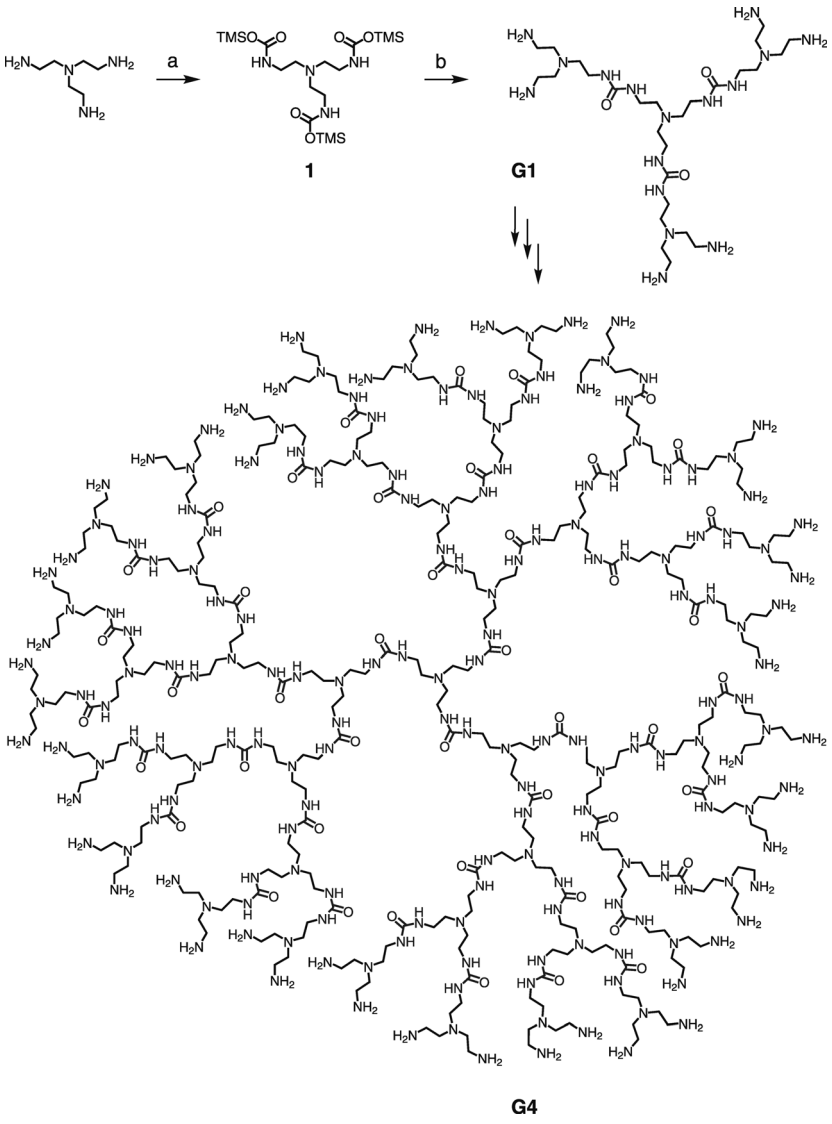

Scheme 1. Synthesis of polyurea (PURE) dendrimers with terminal amino groups in supercritical carbon dioxide. Experimental conditions: a) $scCO_2$, BSA, 18.5 MPa, 40 °C, 20 h; b) BSA, TREN, 120 °C, 17 h. TMS = trimethylsilyl.

cenetriol (dithranol), α-cyano-4-hydroxycinnamic acid (CHCA), and sinapinic acid (SA) resulted in no signal.

The available MALDI techniques for polymer analysis still have several limitations (i.e. analysis of some narrow-polydispersity polymers, polycationic materials),[9] and as reported for PAMAM dendrimers the MALDI-TOF method does not provide a straightforward quantification of this type of polymers.[10] The polydispersity of PURE dendrimers was qualitatively studied by gel permeation chromatography (GPC) through the examination of the elution profiles of their aqueous solutions using a Sephacryl HR S-100 column. The mobile phase used was a NaCl solution (0.2 M) and the flow rate adjusted to 0.5 mL min^{-1}. The eluting dendrimers (**G2**–**G4**) were detected using an UV detector at a fixed wavelength ($\lambda = 280$ nm; see the Supporting Information). As expected, the elution volumes of PURE dendrimers were in the order **G2** > **G3** > **G4**. The PURE dendrimers under study showed narrow elution profiles. The column was calibrated with poly(ethylene glycol) (PEG) standards, but a quantitative analysis was discarded, because the hydrodynamic volume of PEG is larger than that of the PURE dendrimers.[11] The hydrodynamic radius of dendrimer **G4** was determined experimentally in aqueous solution at 25 °C by dynamic light scattering, and it was found to be (1.73 ± 0.02) nm, which is really close to the one reported by Sagidullin et al.[12] for a generation-4 PAMAM dendrimer with terminal hydroxy groups in methanol.

In our studies we found that PURE dendrimers also show high blue fluorescence in aqueous solution. We investigated the influence of the pH value on the fluorescence intensity of PURE dendrimers. Figure 1 shows a consistent increase of the fluorescence intensity with decreasing pH value, especially when the pH value is varied from 7 to 2. PURE dendrimers are also stable at less biologically relevant pH values (below 2). This behavior is not so obvious for dendrimer **G1** and in general for dendrimers of all generations for pH values from 7 to 12. However, a remarkable increase of the fluorescence intensity is observed for dendrimer **G4**.

The fluorescence enhancement for low pH values could be related with the protonation of the terminal primary amino groups (pK_a 7–9) and branch points (tertiary amino groups, pK_a 3–6) of the dendrimers.[12] In this particular case, the presence of ureas in the interior region of the dendrimer backbone will also influence the extent of protonation. In general, the protonation of these macromolecules strongly affects the macromolecular conformation; greater molecular charge results in a more expanded and more hydrated polymer conformation as a conse-

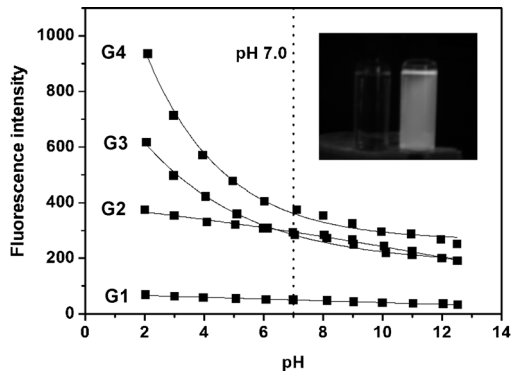

Figure 1. Variation of the fluorescence intensity with pH value for PURE dendrimers in aqueous solution (0.1 mM). The inset shows a photograph of vials containing water (left) and an aqueous solution of dendrimer **G4** (pH 7.0) excited by an UV lamp ($\lambda_{ex} = 366$ nm).

quence of charge–charge repulsions.[13] These results led us to conclude that PURE dendrimers must have an intrinsic blue fluorescence, but we cannot exclude oxidation-triggered enhanced emission processes, especially for higher generations.[4] Moreover, it was found that the fluorescence intensity calculated for one urea unit (at different pH values) does not vary significantly with the dendrimer generation, thus meaning that there is no dendrimer effect on the fluorescence (see the Supporting Information).

To study the applicability of PURE dendrimers for biomedical applications, their cytocompatibility was studied through in vitro studies.

Human fibroblast cells (HFCs) were seeded at the same initial density in 96-well plates, with or without dendrimers to assess their cytotoxicity. After 24, 48, and 72 h, cell adhesion and proliferation was visualized. HFCs adhered and grew in the presence of samples and in the negative control, despite the number of cells decreased over time. In the positive control, no cell adhesion or proliferation was observed. Dead cells with their typical spherical shape can be observed in Figure 2 (the optical micrographs obtained for PURE dendrimers **G1**–**G3** can be found in the Supporting Information).

To further evaluate the biocompatibility of the dendrimers, the MTS assay (MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was also performed for different polymer concentrations (1–10 mg mL^{-1}). The assay showed a significant difference between positive control ($p < 0.05$) and negative control and between cells exposed to samples after 24, 48, and 72 h (Figure 3).

PURE dendrimers are nontoxic for all the tested concentrations (up to 1.7 mM for dendrimer **G4**, a concentration 85-fold higher than the highest concentration tested for PAMAM-G4 analogues,[15] see the Supporting Information), thus suggesting that the PURE dendrimers do not affect cell viability.

Furthermore, the confocal laser scanning microscopy (CLSM) images showed that PURE dendrimers are capable of crossing the cell membrane and the nuclear envelope. As can be seen in Figure 4, the cell nucleus exhibits a high fluorescence emission, and this observation allows us to postulate that these water-soluble dendrimers can be easily

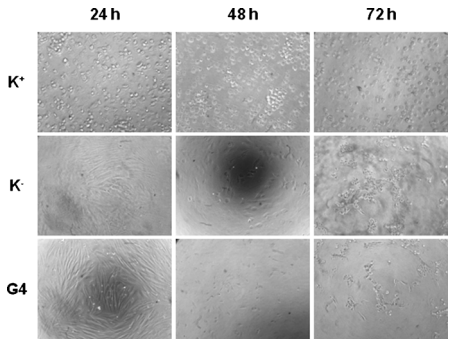

Figure 2. Optical micrographs of human fibroblasts cells after being seeded on dendrimer **G4** (5 mg mL^{-1}) after 24, 48, and 72 h of incubation. K$^+$: positive control (dead cells); K$^-$: negative control (live cells). Original magnification x100.

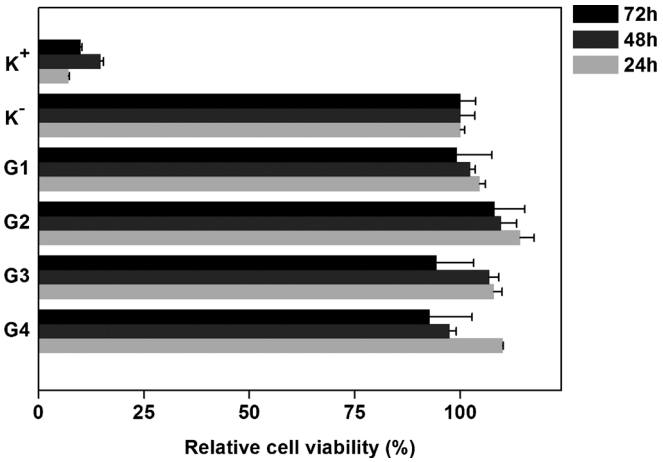

Figure 3. Cellular activities measured by the MTS assay after 24, 48, and 72 h in contact with PURE dendrimers using a concentration of 1 mg mL^{-1}: **G1** (1.6 mM), **G2** (0.59 mM), **G3** (0.27 mM), **G4** (0.17 mM). K$^+$: positive control; K$^-$: negative control. The results shown represent the mean ± standard error of the mean of at least three independent experiments.

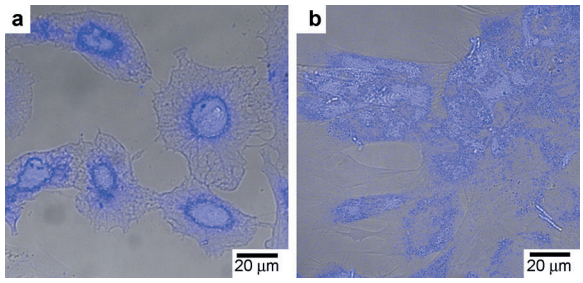

Figure 4. Confocal laser scanning microscopy images of human fibroblast cells incubated with PURE dendrimers (5 mg mL^{-1}): **G1** (a) and **G4** (b).

internalized by cells and therefore used as drug-delivery nanocarriers. Moreover, since PURE dendrimers (especially **G4**) are highly sensitive to changes of the pH value, they can be used as smart probes for the detection of cancer tissues, which are known to have an environment with a lower pH value.[16]

Dendrimers enter into the cells by endocytosis. Cationic dendrimers can be transported by electrostatic interactions through the cell membrane causing its disruption,[17] but endocytic uptake has also been observed.[18]

The transport mechanism of PURE dendrimers into different cell lines is currently under investigation.

In conclusion, we report herein a simple, economic, efficient, and clean synthetic method for the construction of biocompatible and biodegradable water-soluble dendrimers with ureas within the interior and amino groups on the periphery. The dendrimers show a pH-dependent intrinsic blue fluorescence at very low concentrations. They also present an absence of acute cytotoxic effect for HFCs. Endocytic uptake (including nucleus) was observed without disruption of the cell membrane, thus showing a high

potential for biomedical applications. Additional studies are now being pursued with postfunctionalized derivatives.

Experimental Section

Synthesis of PURE dendrimers:[19] a 33 mL stainless-steel high-pressure cell was loaded with TREN (0.5 mL, 3.34 mmol), BSA (5.6 mL, 22.9 mmol), and a magnetic stirrer. The reactor was then closed with two aligned sapphire windows and connected to the CO_2 line charged with gas (99.98%) to approximately 0.1 MPa and placed in a thermostated water bath at 40°C. The pressure was finally adjusted to 18.5 MPa by addition of further CO_2 to solubilize the substrates. The reaction was allowed to proceed under a homogenous supercritical phase for 20 h before depressurization. Once vented the cell was cooled to room temperature and opened in order to add more TREN (4 mL, 26.27 mmol) and BSA (5.6 mL, 22.9 mmol). The mixture was kept at 120°C for 17 h under stirring. For the synthesis of PURE dendrimers **G2**, **G3**, and **G4** the same procedure was used, but on the first step TREN was replaced by the dendrimer of the previously synthesized generation (e.g. dendrimer **G1** was used to synthesize dendrimer **G2**). Since dendrimers of all generations were insoluble in CO_2, 1.78 mol% of DMF relatively to CO_2 was added to the reaction mixture.

The crude products were dissolved in water and purified by dialysis, dried under vacuum, and characterized by FTIR; ^{1}H NMR; and ^{13}C NMR spectroscopy as well as by MALDI-TOF and GPC.

Proliferation of HFCs in the presence of PURE dendrimers **G1**–**G4**: HFCs were seeded in 96-well plates at a density of 5×10^4 cells/well with Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with fetal bovine serum (FBS), for 24 h. After that, the medium was removed; the dendrimers were dissolved in DMEM-F12 at different concentrations (1, 5, and 10 mg mL^{-1}) and placed in contact with cells for 24 h. Cell proliferation was evaluated at 24, 48, and 72 h. Cell growth was monitored using an Olympus CX41 inverted light microscope (Tokyo, Japan) equipped with an Olympus SP-500 UZ digital camera.

Characterization of the cytotoxic profile of PURE dendrimers **G1**–**G4**: HFCs (5×10^4 cells/well) were seeded in a 96-well plate and cultured with DMEM-F12 at 37°C under a 5% CO_2 humidified atmosphere. Then, different concentrations of dendrimers (1, 5, and 10 mg mL^{-1}) were added, and the mitochondrial redox activity of the viable cells was assessed through the reduction of the MTS into a water-soluble brown formazan product as previously described.[20b,21] Wells containing cells in the culture medium without materials were used as negative control. EtOH 96% was also added to some wells, to be used as a positive control.

Confocal laser scanning microscopy (CLSM): To evaluate the entrance of dendrimers into the cells, HFCs were seeded and grown in DMEM-F12 containing 10% FBS, on glass-bottomed coverslips coated with collagen. Then, cells were washed with phosphate-buffered saline (PBS), and subsequently 5 mg mL^{-1} of different dendrimers were added to each well.[15] After four hours, CLSM was used to visualize the intracellular localization of the dendrimers. Confocal and bright field images were obtained with a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, USA) equipped with a plane-apocromat 63x/DIC objective.

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[1] *Dendrimers in Medicine and Biotechnology—New Molecular Tools* (Eds.: U. Boas, J. B. Christensen, P. M. H. Heegaard), The Royal Society of Chemistry, UK, **2006**.
[2] a) *Dendrimer-Based Medicine* (Eds.: I. J. Majoros, J. R. Baker, Jr.), Pan Stanford Publishing, Singapore, **2008**. For recent reviews see: b) D. Astruc, E. Boisselier, C. Ornelas, *Chem. Rev.* **2010**, *110*, 1857–1959; c) M. A. Mintzer, M. W. Grinstaff, *Chem. Soc. Rev.* **2011**, *40*, 173–190.
[3] a) J.-S. Lee, J. Huh, C.-H. Ahn, M. Lee, T. G. Park, *Macromol. Rapid Commun.* **2006**, *27*, 1608–1614; b) G. Jiang, Y. Wang, X. Sunb, J. Shen, *Polym. Chem.* **2010**, *1*, 618–620.
[4] S.-Y. Lin, T.-H. Wu, Y.-C. Jao, C.-P. Liu, H.-Y. Lin, L.-W. Lo, C.-S. Yang, *Chem. Eur. J.* **2011**, *17*, 7158–7161.
[5] X. Feng, L. Liu, S. Wang, D. Zhu, *Chem. Soc. Rev.* **2010**, *39*, 2411–2419.
[6] M. J. Fuchter, C. J. Smith, M. W. S. Tsang, A. Boyer, S. Saubern, J. H. Ryana, A. B. Holmes, *Chem. Commun.* **2008**, *18*, 2152–2154.
[7] *Chemical Synthesis using Supercritical Fluids* (Eds.: P. G. Jessop, W. Leitner), Wiley-VCH, Weinheim, **1999**.
[8] For the synthesis of hyperbranched aliphatic polyureas see: a) S. Rannard, N. Davis, *Polym. Mater. Sci. Eng.* **2001**, *84*, 2. For the synthesis of hyperbranched aromatic polyureas see: b) A. V. Ambade, A. Kumar, *J. Polym. Sci. Part A* **2001**, *39*, 1295–1304.
[9] a) *MALDI MS—A Practical Guide to Instrumentation, Methods and Applications* (Eds.: F. Hillenkamp, J. Peter-Katalinic), Wiley-VCH, Weinheim, **2007**; b) L. Fernandes, R. Rial-Otero, M. Temtem, C. V. de Macedo, A. Aguiar-Ricardo, J. L. Capelo, *Talanta* **2008**, *77*, 882–888.
[10] J. Peterson, V. Allikmaa, J. Subbi, T. Pehk, M. Lopp, *Eur. Polym. J.* **2003**, *39*, 33–42.
[11] M. El-Sayed, M. F. Kiani, M. D. Naimark, A. Hikal, H. Ghandehari, *Pharm. Res.* **2001**, *18*, 23–28.
[12] A. Sagidullin, B. Fritzinger, U. Scheler, V. D. Skirda, *Polymer* **2004**, *45*, 165–170.
[13] M. H. Kleinman, J. H. Flory, D. A. Tomalia, N. J. Turro, *J. Phys. Chem. B* **2000**, *104*, 11472–11479.
[14] W. Chen, D. A. Tomalia, J. L. Thomas, *Macromolecules* **2000**, *33*, 9169–9172.
[15] K. Fant, E. K. Esbjörner, A. Jenkins, M. C. Grossel, P. Lincoln, B. Nordén, *Mol. Pharm.* **2010**, *7*, 1734–1746.
[16] L. E. Gerweck, K. Seetharaman, *Cancer Res.* **1996**, *56*, 1194–1198.
[17] S. Hong, A. U. Bielinska, A. Mecke, B. Keszler, J. L. Beals, X. Shi, L. Balogh, B. G. Orr, J. R. Baker, Jr., M. M. Banaszak Holl, *Bioconjugate Chem.* **2004**, *15*, 774–782.
[18] a) R. Jevprasesphant, J. Penny, D. Attwood, A. D'Emanuele, *J. Controlled Release* **2004**, *97*, 259–267; b) K. M. Kitchens, A. B. Foraker, R. B. Kolhatkar, P. W. Swaan, H. Ghandehari, *Pharm. Res.* **2007**, *24*, 2138–2145; c) M. El-Sayed, C. A. Rhodes, M. Ginski, H. Ghandehari, *Int. J. Pharm.* **2003**, *265*, 151–157.
[19] The apparatus for polymerization is described elsewhere: C. Veiga de Maced, M. S. Silva, T. Casimiro, E. J. Cabrita, A. Aguiar-Ricardo, *Green Chem.* **2007**, *9*, 948–953.
[20] a) J. Maia, M. Ribeiro, C. Ventura, R. Carvalho, I. Correia, M. Gil, *Acta Biomater.* **2009**, *5*, 1948–1955; b) M. Ribeiro, A. Espiga, D. Silva, P. Baptista, J. Henriques, C. Ferreira, J. Silva, J. Borges, E. Pires, P. Chaves, *Wound Repair Regen.* **2009**, *17*, 817–824.
[21] A. Palmeira-de-Oliveira, M. Ribeiro, R. Palmeira-de-Oliveira, C. Gaspar, S. Costa-de-Oliveira, I. Correia, C. Pina Vaz, J. Martinez-de-Oliveira, J. Queiroz, A. Rodrigues, *Gynecol. Obstet. Invest.* **2010**, *70*, 322–327.

