DOI: 10.1002/adem.201180063

Influence of Silicification on the Structural and Biological Properties of Buffer-Mediated Collagen Hydrogels**

By *Sandrine Quignard, Guillermo J. Copello, Carole Aimé, Isabelle Bataille, Christophe Hélary, Martin F. Desimone* and *Thibaud Coradin**

A buffer-mediated gelation route for collagen hydrogels that allows the formation of homogeneous composite and hybrid materials with various silica sources (i.e., colloidal silica and soluble silicates) at high concentration (up to 25×10^{-3} M) is described. Most significant improvement in rheological properties and proliferation of primary adult human dermal fibroblasts was obtained for the silicate-based hybrid materials. A similar trend was observed in composite materials incorporating 14 nm SiO_2 nanoparticles, although to a much lesser extent, whereas larger colloids (80 and 390 nm) did not significantly impact mechanical stability and cell behavior. Modification of 80 nm particles surface with amine groups weakens the collagen-mineral interface, resulting in the decrease of material stability and leading to particle aggregation during the course of cell proliferation experiments.

The association of collagen with silica within composite (obtained by addition of pre-formed colloids) and hybrid (obtained by in situ silica polymerization) hydrogels has recently attracted a great deal of attention to design biomaterials exhibiting improved mechanical properties with targeted applications for bone repair and biological dressings.[1–3] A variety of preparation methods have been described, either from using acidic solutions of collagen triple helices or from collagen fibrils at neutral pH, associated with silicon alkoxide, silicates, silica nanoparticles, or bioglasses.[1–4] However, several limitations exist in the composition range of available materials: increasing viscosity of collagen solutions with concentration, faster gel formation kinetics with silica concentration, inhibition of collagen fibrillogenesis by silica precursors and strict neutral pH conditions if cell immobilization is targeted.[5] In this context, we have recently shown that soluble silica and silica nanoparticles (12 nm, Si12) at a 10×10^{-3} M concentration could be associated with $3\,mg \cdot mL^{-1}$ collagen solutions to obtain homogeneous hybrid and composite materials suitable for primary adult human dermal fibroblast (HDFa) cells 3D immobilization.[2] Our motivation for the present work was: i) to further improve the mechanical properties of these systems by identifying suitable conditions to induce collagen triple helices self-assembly at a higher initial collagen concentration in the presence of several silica sources at higher silica concentration and ii) to clarify the effect of silica addition on cell/material interactions. This could be achieved by neutralization of collagen/silica solutions via specific buffer addition, resulting in homogeneous materials with up to 10-fold increased storage modulus and a wide range of cellular responses from improved short-term adhesion to enhanced long-term proliferation of HDFa in vitro.

The traditional routes for the preparation of collagen hydrogels by neutralization of acidic triple helices solutions depend on the initial protein concentration. At high final collagen content ($>5\,mg \cdot mL^{-1}$), the collagen solution is neutralized under ammonia vapor[6] but this process is not compatible with cell incorporation and the final pH remains difficult to control.[5] For final concentration up to $5\,mg \cdot mL^{-1}$, the initial process described by Bell relies on addition of phosphate buffer saline (PBS) and NaOH to the collagen

[*] *Dr. T. Coradin, Dr. S. Quignard, Dr. C. Aimé, Dr. C. Hélary UPMC Univ Paris06, CNRS, Chimie de la Matière Condensée de Paris*
Collège de France, 11 Place Marcelin Berthelot, F-75005 Paris, (France)
E-mail: thibaud.coradin@upmc.fr
Dr. G. J. Copello, Dr. M. F. Desimone
IQUIMEFA-CONICET, Facultad de Farmacia y Bioquimica Universidad de Buenos Aires, 1113 Buenos Aires, (Argentina)
Dr. I. Bataille
INSERM, U698, Bioingénierie des Polymères Cardiovasculaires, Institut Galilée, Univ Paris 13, 99 av JB Clément, F-93430 Villetaneuse, (France)

[**] *G. J. C. thanks CONICET for funding his travel to Paris. Funding from the ANR Bone Printing project is acknowledged. We thank Dr. Marie-Madeleine Giraud-Guille and J. Silvent for fruitful discussions and N. Abdoul-Aribi for assistance in chemical analyses. Supporting Information is available from the Wiley Online Library or from the author.*

 © 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

solution (17×10^{-3} M acetic acid),[7] and was recently improved by increasing the concentration of the collagen stock solution.[8] However, our first attempts to apply this technique to obtain $5\,mg \cdot mL^{-1}$ collagen hydrogels in the presence of silicate or Si12 additive at concentration higher than 10×10^{-3} M were unsuccessful due to a combination of fast silica gelation, inhomogeneity of resulting materials and strong variations in the final pH, suggesting that the PBS buffering ability is not able to counterbalance the effect of local pH increase upon NaOH addition.

To overcome these problems, we explored a buffer-mediated neutralization route, i.e., avoiding NaOH addition. We used phosphate buffer (PB) instead of PBS to avoid the presence of NaCl that is known to speed up silica gelation reaction. However high PB molarity (0.5 M) was required when the silica concentration was above 10×10^{-3} M, which again led to fast silica gelation and inhomogeneous materials. We therefore turned our attention to the use of Tris–HCl buffer, another common buffer for protein studies. Addition of Tris–HCl (pH 7.5) at a 1 M concentration allowed the slowing down of the gelation reaction of pure collagen solutions at $6\,mg \cdot mL^{-1}$ to 1 h, resulting in a final $5\,mg \cdot mL^{-1}$ collagen hydrogel. In these conditions, it was also possible to add silicate or Si12 at 25×10^{-3} M and to perform the reaction at room temperature, decreasing initial collagen viscosity and therefore favoring mixture homogeneity, without modifying neither the final pH nor the gelation kinetics. Such a difference between PB and Tris–HCl can be mainly attributed to the fact that phosphate ions are known as kosmotrope anions[9] that should therefore favor collagen self-assembly whereas neither Cl^- nor Tris–H^+ exhibit similar interactions with proteins. It is worth noting that Tris–HCl was already used for collagen gel formation[1] but starting from fibrillar collagen and not from triple helices.

Such an approach could also be extended to other silica particles. Silica particles of 80 nm (Si80) and 390 nm (Si390) diameter were prepared from tetraethoxysilane by the Stöber process to investigate the effect of particle size, together with 80 nm-particles grafted with amino-propyltriethoxysilane providing a positive charge at neutral pH ($Si80NH_2$).[10] These materials were first examined by SEM (Figure 1). The pure collagen hydrogels consist of a fibrillar porous network (Figure 1a). Addition of silicates, Si12 and Si80 (Figure 1b) does not significantly modify this structure. An increase of fiber diameter is apparent for Si390 colloids that are clearly visible on collagen fibers (Figure 1c) and this phenomenon is more significant in the presence of $Si80NH_2$ (Figure 1d).

Chemical characterization of the hydrogels was performed by a combination of FTIR, TGA, and EDX analyses. Typical results are presented in Figure S1 (in the Supporting Information) for silicate- and Si12-collagen materials. The presence of silica could be ascertained by triply degenerated stretching vibration mode of the $[SiO_4]$ tetrahedron ($1100\,cm^{-1}$) and the inter-tetrahedral Si–O–Si bending vibration mode ($800\,cm^{-1}$),[11] whereas the signature of the collagen backbone could be identified especially by amide bands (Amide II, $1640\,cm^{-1}$; Amide I, $1550\,cm^{-1}$). TGA shows that collagen hydrogel (including contribution of Tris–HCl buffer) degradation mainly occurs in the 200–600 °C temperature range with an additional contribution of silica dehydration and condensation in the 550–750 °C range.[12,13] The obtained silica dry weight content was estimated to be 15 wt%, in good agreement with the theoretical one calculated from the initial protein–silica mixture (15 mg SiO_2, 5 mg collagen, 80 mg Tris–HCl). Finally EDX analyses coupled to SEM observation yield a Si:C weight ratio of 0.25 ± 0.05, once again in good agreement with the initial solution content (Si:C $\approx$ 0.3).

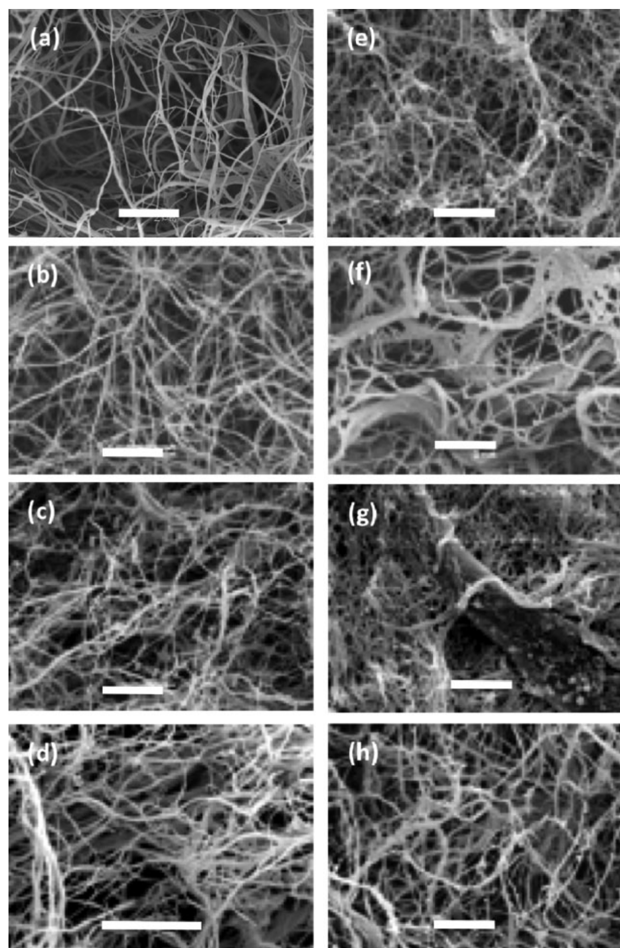

Fig. 1. SEM images of hydrogel surface after 1 day (cell-free) and 21 days (HDFa culture) for (a,e) pure collagen, (b,f) collagen-Si80 composites, (c, g) collagen-Si390 composites, and (d,h) collagen-Si80NH$_2$ composites (scale bar = 5 μm).

The mechanical behavior of the different materials after 24 h, as obtained from rheological measurements, was also compared. As seen in Figure 2, storage modulus G' of the collagen network was not modified by the presence of the largest (Si80 and Si390) colloids but slightly (2-fold) increased in the presence of Si12 and is ten time larger in the presence of silicates. Interestingly, the positive $Si80NH_2$ particles tend to decrease the collagen hydrogel cohesion. These data are confirmed by variations in loss moduli G'' values. These results are in good agreement with expected behavior of nanocomposites where the moduli increase with larger and

 © 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

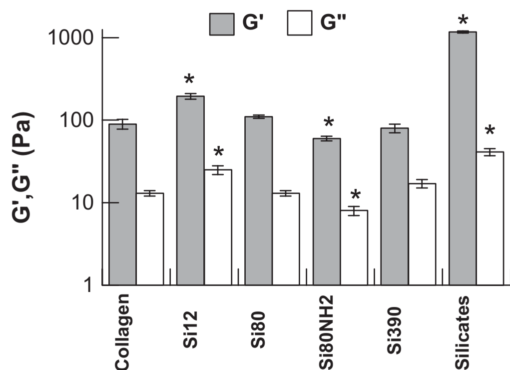

*Fig. 2. Storage G′ and loss G″ moduli for the different materials. Results are expressed as mean ± SD from triplicates experiments. * indicates statistical significance ($p < 0.05$) compared to collagen hydrogels from Wilcox test.*

stronger polymer–filler interfaces, i.e., smaller particle size (i.e., Si12) and attractive electrostatic interactions (compare Si80 and $Si80NH_2$). Silicate-based hybrid materials represent a specific situation as they consist of interpenetrated networks of bio-organic and inorganic polymers developing a large surface of interaction, so that silicification can significantly modify the polymer mechanical properties.[14] A comparison with previous reports on silicified collagen hydrogels at $0.66\,mg \cdot mL^{-1}$ final protein concentration and 10×10^{-3} M silica content indicate a 5-fold increase in the storage moduli of pure collagen gel, Si12, and Si80 composites,[2] validating our approach to strengthen collagen hydrogels by increasing both collagen and silica concentration.

Both the modification of collagen gelation conditions and introduction of silica may have a negative impact on cell/hydrogel interfaces. Therefore, to validate these materials as substrate for cell growth, adhesion and proliferation tests of HDFa were performed (Figure 3). For the control collagen gel, cell adhesion slowly increases over 24 h. As a general trend, Si80 particles were the most efficient in favoring cell adhesion over the whole period. Smaller Si12 colloids virtually have no influence compared to the control. Positively charged $Si80NH_2$ and larger particles Si390 showed very similar, overall slightly detrimental effect on cell adhesion. In the case of silicates, the test was unsuccessful after 30 min, due to incomplete silica condensation leading to loose network. Adhesion was then improved over the following 6 h. However, after 24 h, silicate-containing gels showed the lowest cell density. Proliferation tests performed at this time point are in rather good correlation with adhesion data. In the following 3 weeks, collagen hydrogels show increased proliferation, followed by a decrease that may indicate that confluency has been reached and that cell colonization is occurring, allowing a subsequent increase. Over this period, collagen-Si12 hydrogels are the most effective among composite materials in favoring cell proliferation compared to the control. On the average, Si80 and Si390 colloids had minor influence on cell viability. $Si80NH_2$ appears as an intermediate situation inducing a significant improvement of cell proliferation in the short term. Finally, hybrid materials formed with silicates showed a continuous and very sharp increase in viable cells after 2 weeks and, more significantly, 3 weeks.

SEM observations of the hydrogel after 21 days cell culture provide interesting insights on their structure after proliferation. Whereas the pure collagen material is denser due to higher fibril interconnection (Figure 1e), significant fibril aggregation is observed for silicate, Si12 and Si80 (Figure 1f). Such a denser packing of collagen fibrils is also evidenced in Si390 composite and Figure 1g also shows that HDFa cells are locally coated by collagen fibers, in agreement with the fact that hydrogel colonization has started. For $Si80NH_2$, there is no clear sign of further fibril aggregation but the colloids are now visible as aggregates on the collagen network (Figure 1h), suggesting that they have moved during the culture period.

Adhesion mainly depends on the ability of specific proteins, such as integrins, to bind on the hydrogel surface, and therefore depends on surface chemistry and topology.[15] From a biochemical point of view, collagen is a natural substrate for fibroblast adhesion so that the presence of silica can, in principle, only decrease the affinity of binding proteins for the hydrogel surface. However, the introduction of colloidal silica also leads to some modification in the surface topology of the material. In this context, earlier reports studying cell adhesion on silica nanoparticle-coated glass substrates showed that nanostructured surfaces with increasing roughness (i.e., increasing particle size) influence the conformation of cell-binding proteins and the formation of focal adhesion complexes, therefore hindering cell adhesion.[16] However, because all materials were prepared at similar molar content, larger particles are less numerous and exhibit a lower specific surface area than smaller particles. Therefore, Si80 appear as an intermediate case resulting from the balance of

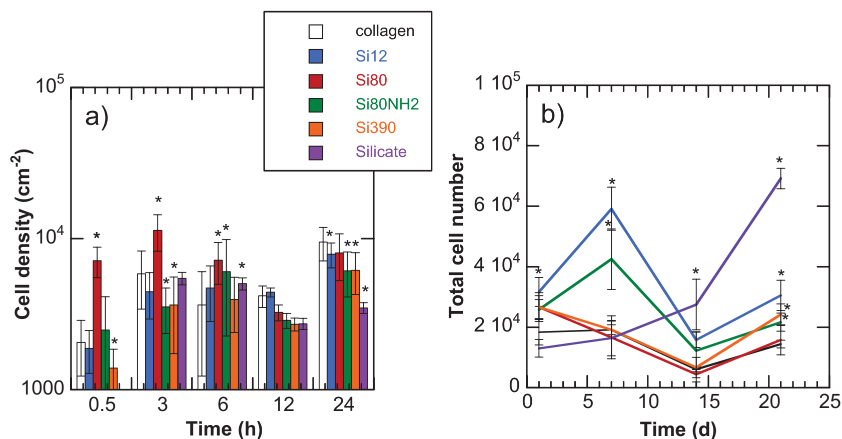

*Fig. 3. (a) Adhesion (as expressed by cell density) and (b) proliferation (as expressed by viable cell number) for HDFa cells seeded on the different materials. Results are expressed as mean ± SD from triplicates experiments. * indicates statistical significance ($p < 0.05$) compared to collagen hydrogels from Wilcox test.*

© 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

surface roughness and particle density. As far as proliferation is concerned, it strongly depends on the ability of cells to anchor into the hydrogel surface. In this case, the mechanical stability of the material becomes a key parameter.[17] Here, when Si12, Si80, and Si390 are compared, the highest proliferation is observed for the more robust gels, i.e., in the presence of Si12. The hybrid materials obtained from silicates show more limited cell adhesion but significantly better cell proliferation, reflecting the important coverage of the collagen surface by the silica network and the strong mechanical stability of the hybrid system. This is in good agreement with recent reports on gelatin-silica cross-linked materials.[18] The effect of particle charge is more difficult to ascertain. Overall, $Si80NH_2$-collagen gels lead to lower cell adhesion but better cell proliferation compared to Si80-collagen gels. Interestingly, our data show that these colloids induce lower moduli and larger collagen fibers, due to electrostatic repulsion between the particles and the protein during gel formation, which may explain lower cell adhesion. In contrast, after 21 days, the main observation is related to particle aggregation that leads to a more open and chemically-heterogeneous surface, exhibiting silica-depleted regions, that may be more favorable for cell anchoring and proliferation on the collagen surface.

As a conclusion, the here-reported method to slow down the formation of collagen hydrogels up to 1 h at a relatively high concentration ($5\,mg \cdot mL^{-1}$) opens the route to design novel composite and hybrid structures without being impaired by rapid gelation times. This interest is validated here in the case of silicification that allows an improvement in rheological properties as well as adhesion/proliferation of HDFa cells compared to pure collagen hydrogels. Overall, silicate and small silica colloids reveal to be the most promising sources for further development of collagen-silica biomaterials. In the future, rational surface functionalization[19] or use of organosilanes[3b,15] may allow further tailoring of the collagen-silica interface to obtain novel 2D, and maybe 3D, cell growth environments. These results should also contribute to further combination of collagen with other sol-gel based metal oxide networks, such as ZrO_2 or TiO_2, of increasing interest in the field of biomaterials, as functional coatings or tissue repair devices.[20]

1. Experimental

1.1. Silica-Collagen Materials Preparation and Characterization

Collagen type I was purified from rat tails and the concentration was estimated by hydroxyproline titration. Silica nanoparticles 12–14 nm in size (Si12, Ludox HS-40) and sodium silicate were purchased from Aldrich. Silica nanoparticles 80 and 390 nm were prepared following the Stöber route. $Si80NH_2$ particles were prepared by reaction of Si80 colloids with aminopropyltriethoxysilane. Particle size was checked by dynamic light scattering and transmission electron microscopy and surface modification ascertained by zeta potential measurements. Silica-collagen materials were prepared by mixing 2.5 mL collagen solution ($10\,mg \cdot mL^{-1}$ in 17×10^{-3} M acetic acid) with 1.7 mL of deionized water followed by addition of 0.25 mL of silica sources (0.5 M in acetic acid, pH 3) and neutralization with 0.55 mL of Tris–HCl (1 M, pH = 7.5). Details for SEM observations and rheological measurements can be found in Ref. [2]. X-ray energy dispersive spectrometry (EDX) was performed using a IXRF system integrated to the SEM apparatus. Chemical analyses were obtained using the IXRF software. Thermogravimetric analyses (TGA) were performed on a NETSCH STA 409PC apparatus under air with a $5\,°C \cdot min^{-1}$ heating ramp. Fourier-transform infra-red (FTIR) spectroscopy was performed on a Perkin Elmer Spectra 400 equipment.

1.2. Adhesion/Proliferation Experiments

Primary adult human dermal fibroblast (HDFa) cells (10^5 corresponding to $30\,000\,cells \cdot cm^{-2}$) were added at the surface of each gel with culture medium. Medium was changed every week and replaced by fresh medium for the proliferation experiments. For adhesion measurements, at different times after the addition of cells (0.5, 3, 6, 12, and 24 h), the gels were rinsed three times with PBS and fixed with 4% paraformaldehyde for 1 h. Samples were again washed three times with PBS, the cells were permeabilized by Triton X-100 (0.05% in PBS-1% BSA) for 10 min and the nuclei were stained with DAPI ($5\,\mu g \cdot mL^{-1}$ in PBS) for 2 min. A count of remaining adherent cells was performed with a fluorescent microscope, over a total of 7 random fields (×10 magnification) for each sample. For proliferation experiments, medium was removed after 24 h, 7 and 21 days and a $5\,mg \cdot mL^{-1}$ solution of MTT in PBS was added to the gels and incubated at 37 °C in a humidified 5% CO_2 air atmosphere for 4 h. Afterwards, MTT solution was removed, the gels were washed three times with water and DMSO was added for 30 min. The optical density was recorded at 570 nm. Readings were converted to cell number using a standard curve. For each condition, sample number was three or more. Statistical analysis was carried out using Wilcox test (for non-parametric distribution) using collagen hydrogel as reference. Differences were considered significant when $p < 0.05$.

Received: July 12, 2011
Final Version: October 1, 2011
Published online: November 2, 2011

[1] a) S. Heinemann, H. Ehrlich, C. Knieb, T. Hanke, *Int. J. Mater. Res.* **2007**, *98*, 603. b) S. Heinemann, C. Heinemann, H. Ehrlich, M. Meyer, H. Balzer, H. Worch, T. Hanke, *Adv. Eng. Mater.* **2007**, *9*, 1061. c) S. Heinemann, C. Heinemann, R. Bernhardt, A. Reinstorf, B. Nies, M. Meyer, H. Worch, T. Hanke, *Acta Biomater.* **2009**, *5*, 1979.
[2] a) M. F. Desimone, C. Helary, G. Mosser, M. M. Giraud-Guille, J. Livage, T. Coradin, *J. Mater. Chem.* **2010**, *20*, 666. b) M. F. Desimone, C. Helary, I. B. Rietveld, I. Bataille, G. Mosser, M. M. Giraud-Guille, J. Livage, T. Coradin, *Acta Biomater.* **2010**, *6*, 3998.
[3] a) B. Marelli, C. E. Ghezzi, J. E. Barralet, A. R. Boccacini, S. N. Nazhat, *Biomacromolecules* **2010**, *11*, 1470. b) S. Chen, A. Osaka, N. Hanagata, *J. Mater. Chem.* **2011**, *21*, 4332.
[4] a) Y. Ono, Y. Kanekiyo, K. Inoue, J. Hojo, M. Nango, S. Shinkai, *Chem. Lett.* **1999**, *28*, 475. b) D. Eglin, G. Mosser, M. M. Giraud-Guille, J. Livage, T. Coradin, *Soft Matter*

 © 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

2005, *1*, 129; c) D. Eglin, S. Maalheem, J. Livage, T. Coradin, *J. Mater. Sci. Mater. Med.* **2006**, *17*, 161.

[5] a) T. Coradin, M. M. Giraud-Guille, C. Hélary, J. Livage, C. Sanchez, *Mat. Res. Symp. Proc.* **2002**, *726*, Q.5.2.1. b) D. Eglin, K. L. Shafran, J. Livage, T. Coradin, C. C. Perry, *J. Mater. Chem.* **2006**, *16*, 4220.

[6] L. Besseau, M. M. Giraud-Guille, *J. Mol. Biol.* **1995**, *251*, 197.

[7] E. Bell, B. Ivarsson, C. Merrill, *Proc. Natl Acad. Sci. USA* **1979**, *76*, 1274.

[8] C. Hélary, I. Bataille, A. Abed, C. Illoul, A. Anglo, L. Louedec, D. Letourneur, A. Meddahi-Pellé, M. M. Giraud-Guille, *Biomaterials* **2010**, *31*, 481.

[9] R. L. Baldwin, *Biophys. J.* **1996**, *71*, 2056.

[10] W. Stober, A. Fink, E. Bohn, *J. Colloid Interf. Sci.* **1968**, *26*, 62.

[11] A. Gendron-Badou, T. Coradin, J. Maquet, F. Frölich, J. Livage, *J. Non-Cryst. Solids* **2003**, *316*, 331.

[12] C. Gautier, N. Abdoul-Aribi, C. Roux, P. J. Lopez, J. Livage, T. Coradin, *Colloids Surf. B* **2008**, *65*, 140.

[13] J. J. Lim, *J. Biol. Phys.* **1975**, *3*, 111.

[14] J. C. Tan, A. K. Cheetham, *Chem. Soc. Rev.* **2011**, *40*, 1059.

[15] K. Anselme, L. Ploux, A. Ponche, *J. Adhes. Sci Technol.* **2010**, *24*, 831.

[16] a) B. G. Cousins, P. J. Doherty, J. Fink, M. J. Garvey, R. L. Williams, *J. Mater. Sci. Mater. Med.* **2004**, *15*, 353. b) M. S. Lord, B. G. Cousins, Doherty, J. M. Whitelock, A. Simmons, R. L. Williams, B. K. Milthorpe, *Biomaterials* **2006**, *27*, 4856. c) B. G. Cousins, H. E. Allison, P. J. Doherty, C. Edwards, M. J. Garvey, D. S. Martin, R. L. Williams, *J. Appl. Microbiol.* **2007**, *102*, 757. d) A. M. Lipski, C. J. Pino, F. R. Haselton, I.-W. Chen, V. P. Shashtri, *Biomaterials* **2008**, *29*, 3836.

[17] S. Mitragotri, J. Lahann, *Nature Mater.* **2009**, *8*, 15.

[18] O. Mahony, O. Tsigkou, C. Ionescu, C. Minelli, L. Ling, R. Hanly, M. E. Smith, M. M. Stevens, J. R. Jones, *Adv. Funct. Mater.* **2010**, *20*, 3835.

[19] Z. Luo, K. Cai, Y. Hu, L. Zhao, P. Liu, L. Duan, W. Yang, *Angew. Chem. Int. Ed.* **2011**, *50*, 640.

[20] M. Vallet-Regi, M. Colilla, B. Gonzalez, *Chem. Soc. Rev.* **2011**, *40*, 596.

 © 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim