Nonaqueous Synthesis of Macroporous Nanocomposites Using High Internal Phase Emulsion Stabilized by Nanohydroxyapatite

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1. Introduction

One of the most challenging tasks in mimicking the extracellular matrix for tissue engineering purposes is to achieve the 3D architectural features that allow for cell placement, attachment, and proliferation while providing mechanical support and a suitable chemical environment. In this regard, bottom-up emulsion-templating strategies have attracted much attention due to their versatility and simple preparation.[1]

Among them, high internal phase emulsions (HIPEs) are highly viscous systems characterized by an internal phase volume exceeding 74% that is dispersed within a minor continuous phase. [2] Above a critical value, spherical droplets exceed their physical packing limit and adopt a compact polyhedral conformation. Introduction of a polymerizable continuous phase and subsequent extraction of the internal phase affords a porous functional matrix commonly known as poly(HIPEs). Resulting poly(HIPEs) monoliths are nearly exact replicas of their precursor.

Nonaqueous high internal phase emulsions (HIPEs) stabilized by nanohydroxyapatite (NHA)/surfactant hybrids are used as template to prepare interconnected porous monoliths. The use of a sustainable deep eutectic solvent (DES) comprised of urea and choline chloride (UChCl), as the internal phase, enables an efficient interaction of NHA/surfactant at the HIPE interface, which in turn allows for a bottom-up approach to selective interfacial functionalization of poly(HIPE's) voids surface after polymerization of methyl methacrylate continuous phase. UChCl DES is a suitable internal phase for HIPE polymerization thanks to its polarity and viscosity that provides further stabilization of the emulsion precursor. This simple synthetic method produces well-defined functional poly(methyl methacrylate) (pMMA) scaffolds with tunable mechanical properties and exposed NHA at the inner surface. Based upon a preliminary biocompatibility in vivo test, poly(HIPEs) show enhanced biocompatibility in comparison with sterile gauze. Interestingly, pMMA NHA nanocomposite scaffold remains in the tissue after 90 d allowing little ingrowth of cells while causing a normal foreign-body reaction in the rats’ muscle tissue. Interfacial functionalization of well-defined interconnected porous monoliths with nanomaterials via DES-based HIPEs approach is a promising method that encourages further investigation for the synthesis of biodegradable and biocompatible scaffolds nanocomposites for tissue engineering purposes.
emulsion having cavities of approximate diameter of the emulsion droplets with smaller interconnecting pore voids. In most cases, raising the surfactant content produces higher degree of interconnecting secondary porosity, whereas decreasing it produces closed pore structures.[3] Changing the internal to continuous phase volume ratio can also modulate openness. Furthermore, tertiary porosity may be induced by inducing phase separation during polymerization through the addition of non-polymerizable materials to the continuous phase. The sum of all these elements adding to the monomer and polymerization techniques selection facilitates the design of functional interconnected scaffolds for specific needs,[4] making poly(HIPEs) very attractive for tissue engineering applications.[5]

Not only can the morphological and mechanical characteristics of polyHIPEs be modulated, but also their chemical nature and most importantly their surface functionality can do so to culture specific cell types. For instance, it is possible to integrate nanoparticles in place of surfactants that allocate in the HIPE surface bringing stability to the systems.[6] These emulsions are termed Pickering HIPEs; they are notably resilient against coalescence due to nanoparticles tendency to adsorb quasi-reversibly to the oil-water interface forming denser barriers.

Recently reported, the strategy of bringing together surfactant and particle HIPE stabilization has created a new opportunity to integrate Pickering HIPEs' stability and functionality to traditional surfactant HIPEs' pore tunability.[7] Such control becomes crucial to design functional scaffolds that suit specific requirements for bone tissue replacements,[8] for example, having bioactive inorganic components like hydroxyapatite (HA).

Attempts to integrate HA to biocompatible poly(HIPE) scaffolds include the synthesis of injectable HIPEs with unique rheological properties that cure in situ upon redox initiation.[9] In another study, Wang and co-workers have developed biodegradable poly(HIPEs) functionalized by poly(L-lactic acid)-grafted HA particles via Pickering HIPEs and solvent evaporation,[10] while with the aid of bovine serum albumin and calcium alginate, HA particles were incorporated into poly(e-caprolactone) poly(HIPE).[11] Bokhari et al., on the other hand, improved the osteoblast differentiation by perfusing a peptide solution into the nano HA-containing poly(HIPE).[12] More recently, poly(HIPE)-HA composite was structured by using direct-write UV stereolithography on photocurable HIPE precursor to produce composite scaffolds with multiscale porosity.[13]

Altogether, O/W or W/O HIPEs involved either aqueous phases with a variety of salts and other substances including phosphoric acid, or volatile organic solvents, some of the latter being highly toxic, such as toluene and dichloromethane.[10a,11,14] Besides the laborious, multistep, or sophisticated techniques and exhaustive washing with organic solvents, the integration of HA to these poly(HIPEs) has been restricted in some cases[10] to its previous functionalization, thus decreasing its possible bioavailability at the surface of poly(HIPEs) cavities.[15]

Deep-eutectic solvents (DESs) as nonaqueous media represent a green alternative for the synthesis of different types of poly(HIPEs).[16] DESs are a new generation of designer solvents comprised of a eutectic mixture formed through association of hydrogen bond donors and ammonium or phosphonium salts (the DES under study are of type III, i.e., composed by an ammonium salt and a hydrogen bond donor).[17] They share many notable qualities with ionic liquids like high thermal and chemical inertness, negligible vapor pressure, tunable polarity, and the ability to dissolve a wide range of solutes. Developing HIPEs in DESs offers not only a more benign route in terms of the use of toxic solvents and additional components,[18] but also poly(HIPEs) processing (e.g., extracting the internal phase)[16a] prior contacting with living systems becomes easier by using DESs. A more interesting approach is the opportunity to explore experimental conditions at which traditional aqueous HIPEs are barely stable.[7,16,b,d] thus broadening the palette of morphologies available through HIPE polymerization.

In this contribution, a nanohydroxyapatite (NHA)/surfactant hybrid was used to stabilize nonaqueous HIPEs composed by urea-choline chloride (UChCl) DES as internal phase and methyl methacrylate (MMA) monomer as continuous phase. NHA/surfactant hybrid allowed integration of up to 1 wt% of NHA functionalizing the internal voids surface of the resulting poly(HIPE). Herein, determination of biocompatibility of resulting poly(HIPE)-NHA (poly(methyl methacrylate) [pMMA] NHA) nanocomposites was carried out in vivo in rats. For that, the overall inflammatory response of rats to pMMA NHA nanocomposites implanted intramuscularly[19] was determined in histological samples as preliminary indicative of bio-inertness in vivo.

2. Results and Discussion

2.1. Scaffold Characterization

pMMA was chosen as a matrix for biocompatibility testing[20] and further characterization due to their precedence in the use of dental prosthetics, bone cement, contact and intraocular lenses, screw fixation in bone, filler for bone cavities, and vertebrae stabilization in osteoporotic patients.[21] The first step taken prior to any further characterization was the optimization of HIPEs' surfactant and NHA content through visual stability upon inversion of their container, followed by mechanical testing and surface hydrophobicity of resulting poly(HIPEs).

Low concentrations of the surfactant Cithrol (between 6 and 8 wt%) had the highest crush strengths while concentrations beyond 8 wt% resulted in poor mechanical properties (Figure 1). Ultimately, 7 wt% was chosen because it was the surfactant concentration previously shown to have higher stability.[16a,c] Additionally, pMMA poly(HIPEs) with 7 wt% presented a relatively lower elastic modulus than 6 and 8 wt% making it slightly less stiff. Elasticity is highly desirable to ensure that materials meet the requirements for implants in nonload bearing applications (overall compressibility of around 3–4 MPa).[22]

Next, the amount of NHA to be used in the in vivo studies was optimized to ensure the best performance during the implant. In general, it was observed that the introduction of NHA onto the poly(HIPEs) matrix significantly decreased their crush strength. However, loading between 0.2 and 1.0 wt% did not show significant changes in their crush strength. Similarly, a decrease in the elastic modulus was observed in all
pMMA NHA poly(HIPEs) suggesting higher elasticity. In the end, 1.0 wt% was chosen due to its similar properties to other pMMA NHA poly(HIPEs) regardless of NHA loading and because similar amounts of NHA present in the matrix would serve as osteoconductive surface for cell development during a future bone tissue’s healing process.\[^{[9,23]}\]

Contact angle measurements (Figure 2) were performed using two different solvent systems (glycerol and nanopure water) to determine polar ($\gamma_p$), nonpolar ($\gamma_d$), and total ($\gamma_t$) surface energy components. Water contact angle of native pMMA were observed to be 77.7 ± 1.1° while NHA monolith functionalization resulted in higher contact angle values ranging from 118.6 ± 0.2° plateauing at a value of around 130 ± 1.6°, depending on the amount of NHA loading. Figure 2b shows a plot of the water contact angle change from native pMMA monolith to NHA functionalized monoliths. When comparing the parent poly(HIPEs) to the NHA functionalized monoliths, the pure polymer had the highest $\gamma_t$ value of 37.4 ± 0.4 mJ m$^{-2}$. Upon 0.2 wt% of NHA addition, the $\gamma_t$ decreased to 20.2 ± 0.4 mJ m$^{-2}$ to a plateauing value of 12.8 ± 0.8 mJ m$^{-2}$. These results suggest that the inclusion of nanomaterials like NHA may impact the surface roughness therefore making the material more hydrophobic.\[^{[15b,24]}\] despite the HA hydrophilic character reported.\[^{[25]}\] This observation points out that the modification of surface wettability is a consequence of the homogenous integration of NHA functionalizing the poly(HIPE) inner walls as it was the case for a similar approach applied to carbon nanotubes.\[^{[7]}\]

MMA and optimized MMA NHA HIPE microstructures were observed by deconvolution microscopy. Both HIPEs showed tightly packed droplets with polyhedral structures (Figure 3). This suggests a minor degree of destabilization promoted by NHA, where NHA particles modify the type of monomer–surfactant interaction. Finished monoliths appeared as low density white solids taking on the dimensions of their preparatory containers. A study of their physical properties revealed that adding NHA increases poly(HIPE) median pore diameter by ~2 μm when compared to pure MMA poly(HIPEs) while maintaining a similar pore window (Table 1). Overall, pMMA NHA poly(HIPEs) have larger pores but reduced pore interconnectedness.

In addition to tunability of poly(HIPE) mechanical properties through the introduction of nanomaterials at the interface, pore diameter can be modulated in the range of 3–50 µm by the judicious selection of surfactant-to-internal phase ratio, DES nature as internal phase, and monomer type.\[^{[16]}\]

To ensure the NHA presence and structural integrity after polymerization and recovery, XRD patterns were obtained for standard NHA, recovered NHA after its precipitation from UChCl DES, pMMA, and pMMA NHA with 1 wt% (Figure 4). NHA standard presents all major HA diffraction lines, (0 0 2), (1 0 2), (2 1 0), (2 1 1), (1 1 2), (3 0 0), (3 1 0), (3 1 2), (3 2 1), and (0 0 4) at 25.8, 28.1, 29.0, 31.8, 32.9, 33.9, 39.8, 46.7, 49.4, and 55.8° angle 2θ respectively, based on the standard XRD pattern card of HA (JCPDS card no. 09-432).\[^{[26]}\] A close examination of pure NHA standard in comparison to the DES recovered NHA, reveals no changes or shifts in the previously described diffraction pattern values, which is indicative that its crystalline structural integrity is maintained throughout the dispersion in the continuous phase in contact with DES and polymerization process, similarly to HA synthesized in

Figure 2. A) Surface energy characterization of pMMA poly(HIPEs) and B) water contact angle as a function of NHA content.
DES\textsuperscript{[27]} and carbon nanotubes\textsuperscript{[7,28]} On the other hand, the pMMA poly(HIPE) shows a distinct amorphous peak at 19.3° angle 2θ and two distinct minor peaks at 23.0 and 81.7° angle 2θ, respectively. The scaffold nanocomposite presented these polymer signature peaks plus the additional crystalline diffraction lines observed before in both the recovered and standard NHA but with reduced intensity.

These results suggest that NHA particles migrate to the DES monomer interface during the dispersion process and remain housed at the internal wall of the cavities' surface after polymerization and subsequent monolith washing, as observed before for carbon nanotubes\textsuperscript{[7]}

To further assess the chemical composition at the surface of poly(HIPE), ATR–FTIR (Attenuated Total Reflectance–Fourier Transform Infrared) spectra of all samples were obtained. Figure 5 shows the characteristic bands associated to carbonyl group at 1723 cm\(^{-1}\), ester bond at 1134 cm\(^{-1}\), and CH bending at 652 cm\(^{-1}\) in pMMA poly(HIPE) spectrum. Regarding pMMA NHA, the inclusion of NHA is verified by the presence of the bands at 1044 and 505 cm\(^{-1}\) due to different vibration modes of PO\textsubscript{4}\textsuperscript{3-} groups, which partially overlap with those of pMMA. As previously reported,\textsuperscript{[15b]} NHA identified by the ATR mode of FTIR is indicative of its exposition at the surface of the poly(HIPE) composite as suggested before by the XRD results.

It is worthy to mention that surface hydrophobic/hydrophilic balance has been reported to play a determinant role in the performance of biomaterials, including polymeric scaffolds and nanocomposites\textsuperscript{[20,29]} Nevertheless, hydrophobic 3D polystyrene scaffolds have been adapted in the growth of hepatocytes and osteoblasts with great success. In the case of osteoblasts, osteoconductivity can be achieved through mineralization by engineering polymeric scaffolds that include HA, regardless of hydrophobicity\textsuperscript{[15a,29,30]}

Dried monolith conversion was determined gravimetrically; both samples had very high conversion to nearly 99% of the original expected monolith mass. However, addition of NHA to the monomer phase resulted in a slight 0.4% reduction in conversion (Table 2). These results are promising, as they suggest no alteration of the product during the polymerization process, which can be done into virtually any shape with potential scalability and little to no change in preparation methodology.

Thermogravimetric analysis (TGA) of all poly(HIPEs) showed thermal decomposition having an onset point beyond 220 °C (Table 2), in agreement with acrylate-based poly(HIPEs) previously reported\textsuperscript{[13]} In general, all poly(HIPEs) retained negligible moisture and there was little change in temperature decomposition and the amount of water retained from pure pMMA to pMMA NHA. Furthermore, no swelling of monoliths was found when soaked in phosphates buffer at physiological pH.

**2.2. Preliminary Biocompatibility Test in Vivo**

So far, biocompatibility of a variety of poly(HIPEs) has been demonstrated by in vitro tests using different cells type\textsuperscript{[1,2]} To the best of our knowledge, here for the first time, a preliminary in vivo biocompatibility test of a polyHIPE was performed in rats.

In the groups of rats with poly(HIPEs) implanted that were sacrificed after challenge at 7, 30, and 90 d, progressive tissue infiltration of inflammatory cells was detected as normal response for a foreign-body material\textsuperscript{[19b,32]} Macroscopic exploration of the animals and tissue samples from the implanted sites during collection of tissue, indicate that during the first 7 d an acute inflammatory response is established for both pMMA and pMMA NHA implants (Figure 6B). However sterile gauze showed, besides signs of acute inflammatory response, morphological alterations at the adjacent tissue at the site within the same time frame (Figure 6A). It can be observed in Figure 7 that the porous pMMA NHA implant remained integral at the different stages of the test, where a complete adhesion to the surrounding muscle tissue

**Table 1.** Morphological properties summary for pMMA and pMMA-NHA (polyHIPEs).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific surface area ( [m^2 \cdot g^{-1}] )</th>
<th>Drop diameter ( [\mu m] )</th>
<th>Pore diameter ( [\mu m] )</th>
<th>Pore window ( [\mu m] )</th>
<th>Deg. of openness ( [%] )</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMMA</td>
<td>1.7 ( \pm 0.5 )</td>
<td>7.4 ( \pm 2.7 )</td>
<td>6.3 ( \pm 2.4 )</td>
<td>1.8 ( \pm 0.6 )</td>
<td>23.2</td>
</tr>
<tr>
<td>pMMA-NHA</td>
<td>Not determined ( Na^a )</td>
<td>8.6 ( \pm 1.5 )</td>
<td>8.7 ( \pm 1.8 )</td>
<td>1.5 ( \pm 0.2 )</td>
<td>8.6</td>
</tr>
</tbody>
</table>

\( ^a\)Not determined.
by a tissue interface is evident. Porosity, along with their mechanical and surface characteristic, supported the progressively infiltration of inflammatory cells during the 90 d of the test. The presence of poly(HIPEs) following 30 d suggest that these have not been reabsorbed by tissue or destroyed by the immune system (Figure S12, Supporting Information), which provides the possibility to sustain a protect implanted cell previously cultured on the poly(HIPE). After 90 d, a chronic foreign-body inflammatory process was observed in the samples with both types of poly(HIPEs) presenting also cicatrization vestiges which could be product of the incision performed in the area of implantation (Figure 7 and Figures S13 and S14, Supporting Information).

It is well documented that nonresorbable PMMA microspheres used in facial plastic surgery field, elicit low-grade foreign body reaction postinjection causing the presence of lymphohistiocytic inflammation and collagen surrounding the PMMA microspheres in human tissue (e.g., abdomen) or dog larynx.\cite{33} which resemble the immunological response invoked by PMMA poly(HIPEs) monoliths in the rats muscle.

The formation of fibrous tissue and proliferation of macrophages and polymorphonuclear cells is accepted as a normal immune response caused by a foreign implanted material that together with the absence of necrotic tissue, can be considered as sign of biocompatibility.\cite{34} It is noteworthy that none of the poly(HIPE) implants of either type after 90 d exhibited a fibrous granuloma or necrosis as was the case for sterile gauze (Figure 7A, Figures S7 and S8, Supporting Information). The inflammatory response in the presence of both types of poly(HIPEs) (pMMA and pMMA NHA) suggests that the level of tolerance to both materials as foreign matter to muscle tissue and measure of biocompatibility, is acceptable to allow the growth and settlement of cells in rats.

3. Conclusions

Taking advantage of a sustainable nonaqueous DES, a series of macroporous scaffolds (pMMA and pMMA functionalized with NHA) were prepared via a simple HIPE polymerization. Stable HIPEs were prepared through stabilization with NHA and surfactant mixtures using UChCl DES as internal phase. Optimization of NHA content was made based on the stability of the HIPE precursor and the mechanical properties of the resulting poly(HIPEs). Overall, there was little change in conversion and thermal properties when comparing pure pMMA poly(HIPE) to pMMA NHA poly(HIPE) nanocomposites. However, their finer morphological features were affected by the inclusion of NHA into the polymeric matrix where a slight increase in pores size was observed. Remarkably, exposition of NHA at the surface of the poly(HIPE) was achieved by means of its interaction at the monomer/DES interface. Upon polymerization, NHA remained functionalizing the interconnected voids of the scaffold, thus modifying the resulting hydrophobic/hydrophilic balance of the nanocomposite surface. These features however, did not impact preliminary biocompatibility results in vivo after implantation in muscular tissue of rats as both, pMMA and pMMA NHA, exhibited similar enhanced biocompatibility when compared with sterile gauze. Furthermore, 90 d after implantation, poly(HIPEs) scaffolds were still observable, hence holding the possibility to sustain a protect implanted cell previously cultured on the poly(HIPEs).

Table 2. Summary of NHA poly(HIPE) thermal properties.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( T_d ) 2% [°C]</th>
<th>( T_d ) 5% [°C]</th>
<th>% ( \text{H}_2\text{O} ) retained</th>
<th>Conversion: gravimetry [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMMA</td>
<td>225</td>
<td>260</td>
<td>0.16</td>
<td>99.1</td>
</tr>
<tr>
<td>pMMA-NHA</td>
<td>223</td>
<td>253</td>
<td>0.20</td>
<td>98.7</td>
</tr>
</tbody>
</table>
4. Experimental Section

All chemical was purchased from Sigma-Aldrich, unless stated otherwise. The continuous phase (20 vol%) was prepared by dissolving 2.0 mol% AIBN (Azobisisobutyronitrile) with respect to the total concentration of C=C reactive groups in a 2:1 monomer (MMA) to crosslinker (EGDMA) molar ratio to a surfactant mixture. The amount of surfactant used was 7 wt% (Cithrol from Croda Ltd.) with respect to the total weight of the emulsion.\textsuperscript{7,16a} Next, 1 wt% NHA (Sigma-Aldrich, average particle size <200 nm) with respect to the continuous phase was added to the monomer/crosslinker/surfactant mixture and vortexed at 3200 rpm for 20 min to ensure homogenous mixing. To prepare the internal nonaqueous DES phase (80 vol%), choline chloride (ChCl) was recrystallized in ethanol and dried at 90 °C in an oven. A 2:1 molar ratio of urea and ChCl were combined and oven heated to 60 °C until a clear viscous, homogeneous liquid was obtained. HIPEs were prepared by mixing both phases in an 8 mL glass vial and vortexing at 3200 rpm for at least 10 min until homogenous emulsion was obtained. The emulsion did not flow upon the inversion of the vial. HIPEs were polymerized in an oven at 60 °C for 24 h. After polymerization was completed, the internal phase and surfactant were removed by a 12 h ethanol Soxhlet extraction. The resulting monoliths were oven dried at 60 °C until constant weight was reached. Dried monolith conversion was determined gravimetrically. Additionally, to ensure that NHA structural integrity was maintained throughout DES treatment and additional experiment was performed where 1 wt% NHA (Sigma-Aldrich, average particle size <200 nm) with respect to the continuous phase was added to the monomer/crosslinker/surfactant mixture and vortexed at 3200 rpm for 20 min to ensure homogenous mixing. To prepare the internal nonaqueous DES phase (80 vol%), choline chloride (ChCl) was recrystallized in ethanol and dried at 90 °C in an oven. A 2:1 molar ratio of urea and ChCl were combined and oven heated to 60 °C until a clear viscous, homogeneous liquid was obtained. HIPEs were prepared by mixing both phases in an 8 mL glass vial and vortexing at 3200 rpm for at least 10 min until homogenous emulsion was obtained. The emulsion did not flow upon the inversion of the vial. HIPEs were polymerized in an oven at 60 °C for 24 h. After polymerization was completed, the internal phase and surfactant were removed by a 12 h ethanol Soxhlet extraction. The resulting monoliths were oven dried at 60 °C until constant weight was reached. Dried monolith conversion was determined gravimetrically. Additionally, to ensure that NHA structural integrity was maintained throughout DES treatment and additional experiment was performed where 1 wt% NHA was dispersed within U:ChCl DES vortexing at 3200 rpm for at least 10 min where water was used as an antisolvent to extract NHA. NHA was then recovered by vortexing the mixture for 5 min at 4000 rpm. NHA was then freeze-dried and weighed where complete NHA recovery was confirmed through XRD analysis. XRD for powder of NHA standard, recovered NHA, pMMA, and pMMA NHA was performed in a Panalytical Empyrey model with a PANalytical Empyrey model with a PXIcD3D multipurpose mounting system in the range of 2θ from 10 to 100° using a diffractometer with a Cu Kα radiation.

The microstructures of DES-based emulsions were studied using deconvolution microscopy (Leica DM RXA). The morphologies of all poly(HIPE) nanocomposites were investigated by scanning electron microscopy (SEM; JSM 6610 LV) with an accelerating voltage of 10 kV. Samples were platinum coated for 240 s in an inert argon atmosphere at 1 × 10\textsuperscript{−3} mbar (Emitech 550). The average droplet size, pore, and pore window diameters were calculated in sets of 100 using Image analysis software. Additionally, the degree of pore openness was estimated using the equation proposed by Krajnc and co-workers.\textsuperscript{13b} Poly(HIPEs)’ thermal stability was assessed by TGA (2950 thermogravimetric analyzer) in an inert nitrogen atmosphere from 25 to 500 °C with a heating rate of 10 °C min\textsuperscript{−1}. TGA was carried out using 5–10 mg of sample in standard aluminum pans. Thermal analysis was performed using TA Universal Analysis software.

The monoliths’ mechanical properties were evaluated according to the ASTM D1621 in an Instron Model 5996 using a 5 kN load cell using a 4 mm min\textsuperscript{−1} compression rate. Samples were measured in triplicates by compressing to 75% of their initial height and their elastic modulus was determined from the initial linear slope obtained from the stress–strain plot. The stress at yield was recorded to show monoliths compression strength.

The contact angles (CA) were measured using a VCA 2000 CA system (VCA, Billerica, MA) at room temperature. For each measurement a 3 μL drop was allowed to equilibrate on the surface for 1 min prior to measuring the contact angle. Surface energy measurements (γs) and both polar (γp) and dispersive (γd) components were obtained from measurements using glycerol and nanopure water using the Fowkes Equation (1).

\[
\gamma_s^2 = \gamma_p^2 + \gamma_d^2 = \frac{1}{2} \gamma_s (1 + \cos \theta)
\]  

For all calculations each liquid was measured six times on different places on the surface to ensure homogeneity of the material. Data for the liquids are water: γs = 72.8 mJ m\textsuperscript{−2}, γp = 21.8 mJ m\textsuperscript{−2}, γd = 51.0 mJ m\textsuperscript{−2}; glycerol: γs = 64.0 mJ m\textsuperscript{−2}, γp = 34.0 mJ m\textsuperscript{−2}, γd = 30.0 mJ m\textsuperscript{−2}.

The in vivo biocompatibility studies were conducted under the guidelines of the Bioethics’ Committee of the Medicine and Psychology Faculty of Autonomous University of Baja California, Mexico (Registration number 1268/2015-2). Laboratory animal space was conditioned with the acquisition and installation of automated light/dark cycle and temperature controllers required for rat conservation and reproduction. Cages, water dispensers, food, substrate, and broodstock room for albino Wistar rats were also acquired. Rat breeding was synchronized to obtain the required number of individuals with the same weight (~250 g) and similar gender proportions (Table S1, Supporting Information).

Animals were anesthetized with chloroform and were shaved and cleansed with alcohol and iodine at the implant site. A cutaneous incision with muscular separation without cutting the bicep femoris (posterior right member) was made and materials were implanted at each site. As a positive inflammatory response control a rat group with sterile gauze implants was included which is known to cause a severe inflammatory response. Once the material was implanted, the incision was closed with silk sutures, the site was cleansed, and the animals were allowed to recover from the anesthetic (Figures S3 and S4, Supporting Information). Rats were placed in their cages with food and water ad libitum, and without any subsequent treatment until their euthanasia date to collect and preserve their tissue in formaldehyde (Figure S1, Supporting Information).

A histological technique was applied for the inclusion of tissue in paraffin, gradual alcohol dehydration, application of refrigerating agents, and dehydration, application of refrigerating agents, and dehydration, application of refrigerating agents.
Keywords

deep eutectic solvents, high internal phase emulsions, hydroxyapatite, in vivo, scaffolds

Received: January 23, 2017
Revised: March 3, 2017
Published online: