A new category of nanoparticle-based $T_1$ MRI contrast agents (CAs) by encapsulating paramagnetic chelated gadolinium(III), i.e., Gd$^{3+} \cdot $DOTA, through supramolecular assembly of molecular building blocks that carry complementary molecular recognition motifs, including adamantane (Ad) and $\beta$-cyclodextrin (CD). A small library of Gd$^{3+}$-DOTA-encapsulated supramolecular nanoparticles (Gd$^{3+}$-DOTA$\cdot$SNPs) was produced by systematically altering the molecular building block mixing ratios. A broad spectrum of relaxation rates was correlated to the resulting Gd$^{3+}$-DOTA$\cdot$SNP library. Consequently, an optimal synthetic formulation of Gd$^{3+}$-DOTA$\cdot$SNPs with an $r_1$ of 173 s$^{-1}$ mM$^{-1}$ (ca. 4-fold higher than clinical Gd$^{3+}$-chelated complexes at high field strengths) was identified. $T_1$-weighted imaging of Gd$^{3+}$-DOTA$\cdot$SNPs exhibits an enhanced sensitivity with a contrast-to-noise ratio (C/N ratio) ca. 3.6 times greater than that observed for free Gd$^{3+}$-DTPA. A Gd$^{3+}$-DOTA$\cdot$SNPs solution was injected into foot pads of mice, and MRI was employed to monitor dynamic lymphatic drainage of the Gd$^{3+}$-DOTA$\cdot$SNPs-based CA. We observe an increase in signal intensity of the brachial lymph node in $T_1$-weighted imaging after injecting Gd$^{3+}$-DOTA$\cdot$SNPs but not after injecting Gd$^{3+}$-DTPA. The MRI results are supported by ICP-MS analysis ex vivo. These results show that Gd$^{3+}$-DOTA$\cdot$SNPs not only exhibits enhanced relaxivity and high sensitivity but also can serve as a potential tool for diagnosis of cancer metastasis.
liposomes [11,13,15,16], and inorganic nanoparticles (NPs) [17–20] allow dramatically increased Gd^{3+} loading. In some cases, poor water accessibility to the intraparticular Gd^{3+} buried inside the carriers can compromise the performance in T1-weighted MRI CA [21] and this challenge can be tackled by, for example, using porous nanocarriers [8,22] where a water molecule can travel from the bulk water into the interior space. Nevertheless, fine-tuning the balance of the relevant parameters in a synergistic fashion is challenging due to the lack of direct correlations among the parameters, not to mention the unsatisfactory synthetic approach employed for the development of Gd^{3+}-incorporated nanocarriers that are considered to be time-consuming and cumbersome [23]. Therefore, there is a need to establish a method capable of effective optimization of all parameters to achieve high relaxivity enhancement of T1-weighted MRI CAs.

Previously, we introduced a convenient, flexible, and modular self-assembly approach [24] for the preparation of supramolecular NPs (SNPs) by mixing three molecular building blocks, including an adamantane-grafted (Ad-grafted) polyamidoamine dendrimer (Ad-PAMAM), a β-cyclodextrin-grafted (CD-grafted) polyethyleneimine (CD-PEI), and an Ad-grafted polyethylene glycol (Ad-PEG). A variety of SNPs with controllable sizes, surface chemistry, and cross-linking degree of the corresponding hydrogel network in the SNP cores were prepared. We were able to demonstrate that the uniqueness of this supramolecular synthetic approach facilitates the preparations of different SNPs for applications in PET imaging [24], gene delivery [25,26], and photothermal treatment [27]. We set out to further explore such a self-assembled approach to develop Gd^{3+}-DOTA-encapsulated SNPs (Gd^{3+}-DOTA⊂SNPs, Fig. 1) that can be utilized as highly efficient T1-weighted MRI CAs. In our design, Gd^{3+} chelated complex (Gd^{3+}-DOTA) is covalently conjugated onto CD-PEI building block to give Gd^{3+}-DOTA-CD-PEI, which enhances $r_1$ due to a distinct chemical environment with a high Gd^{3+} loading capacity inside the pseudo porous polymer-dendrimer hydrogel network and good water accessibility to the intraparticular space. By altering the mixing ratios among Gd^{3+}-DOTA-CD-PEI and the other two molecular building blocks (Ad-PAMAM and Ad-PEG), a small library of Gd^{3+}-DOTA⊂SNPs can be produced with different sizes and cross-linking degrees of the corresponding hydrogel networks. A systematic variation of SNP sizes and cross-linking degrees results in a broad performance diversity of $r_1$ in the Gd^{3+}-DOTA⊂SNP library due to the respective changes in relaxation parameters. By finding the balance between the material’s properties and $r_1$ performance of the resulting library, Gd^{3+}-DOTA⊂SNPs could serve as a powerful and efficient SNP-based T1-weighted MRI CA for dynamic imaging of biological processes in vivo.

Herein, we demonstrate the self-assembly of a small library of Gd^{3+}-DOTA⊂SNPs from three molecular building blocks — namely, Gd^{3+}-DOTA-CD-PEI, Ad-PAMAM, and Ad-PEG as depicted in Fig. 1. By systematically mixing the molecular building blocks, a broad spectrum of $r_1$ is correlated to the resulting Gd^{3+}-DOTA⊂SNP library by evaluating the relaxation rate. In addition, we use T1-weighted imaging to detect Gd^{3+}-DOTA⊂SNPs in vitro. We monitored lymphatic system uptake of Gd^{3+}-DOTA⊂SNPs in vivo with MRI over time and compared results with ICP-MS analysis of brachial lymph node tissue ex vivo. The results show that Gd^{3+}-DOTA⊂SNPs not only exhibit enhanced relaxivity and high sensitivity but can also serve as a potential tool for diagnosis of cancer metastasis.

2. Materials and methods

2.1. Materials

Reagents and solvents were purchased from Sigma–Aldrich (St. Louis, MO) and used as received without further purification otherwise noted. 1-Adamantanamine (Ad) hydrochloride and β-cyclodextrin (β-CD) were purchased from TCI America (San Francisco, CA). DOTA/CD-grafted branched polyethyleneimine (DOTA-CD-PEI) and Ad-grafted polyethylene glycol (Ad-PEG) were prepared via the method previously reported by our group [24].

2.2. Synthesis of Gd^{3+}-DOTA-CD-PEI

Various quantities of GdCl₃·6H₂O (0.1, 0.2, 0.5, 1.0, 1.2, and 2.0 mg, verified with ICP-MS) were added to a 750-μL solution of DOTA-CD-PEI and the reaction mixture was stirred at rt for 20 min to obtain Gd^{3+}-DOTA-CD-PEI with different Gd^{3+} loading concentration.

2.3. Synthesis of Gd^{3+}-DOTA⊂SNPs

To a 750-μL solution of Ad-PEG (9 mg/mL), various concentrations of Ad-PAMAM (22.5, 15, 11.3, 7.5 mg/mL) in 15-μL DMSO were slowly injected under vigorous stirring. A 750-μL Gd^{3+}-DOTA-CD-PEI solution containing different Gd^{3+} loading concentration (0.32, 0.63, 1.61, 3.22, 3.9 and 6.44 mM, verified with ICP-MS) was sequentially added into the mixture to obtain a collection of Gd^{3+}-DOTA⊂SNPs.

2.4. Dynamic light scattering (DLS)

DLS experiments were performed with a Zetasizer Nano instrument (Malvern Instruments Ltd., United Kingdom) equipped with a 10-mW helium-neon laser (λ = 632.8 nm) and thermoelectric temperature controller. Measurements were taken at a 90° scattering angle.

Fig. 1. Schematic representation of self-assembly approach for preparation of Gd^{3+}-DOTA-encapsulated supramolecular nanoparticles (Gd^{3+}-DOTA⊂SNPs).
2.5. Transmission electron microscope (TEM)

The morphology and sizes of Gd\textsuperscript{3+} DOTA\textsubscript{3} SNPs were examined on a Philips CM 120 transmission electron microscope (TEM), operating at an acceleration voltage of 120 kV. The TEM samples were prepared by drop-coating 2 μL of Gd\textsuperscript{3+} DOTA\textsubscript{3} SNPs solutions onto carbon-coated copper grids. Excess amounts of droplets were removed by filter papers after 45 s. Subsequently, the surface-deposited Gd\textsuperscript{3+} DOTA\textsubscript{3} SNPs were negatively stained with 2% uranyl acetate for 45 s before the TEM studies.

2.6. Zeta potential measurements

Zeta potentials of Gd\textsuperscript{3+} DOTA\textsubscript{3} SNPs were determined by photon correlation spectroscopy using a Zetasizer Nano instrument, (Malvern Instruments, Malvern, Worcestershire, UK). The measurements were performed at 25 °C with a detection angle of 90°, and the raw data were subsequently correlated to Z average mean size using a cumulative analysis by the Zetasizer software package.

2.7. ICP-MS analysis

Samples (20 μL) were diluted to a total volume of 2.00 mL using aqueous 2% nitric acid. 20 μL of a 5-ppm aqueous indium ion solution (ICP-MS grade) was then added to each tube as an internal standard and the contents vortexed vigorously. ICP measurements were then conducted on an Agilent 7500 Series ICP-MS in helium collision gas mode. Reported measurements of gadolinium in each sample represent the average of 3 measurements, and are back-calculated to offset the initial dilution. The individual measurements are typically obtained with a relative standard deviation of approximately 3% or less per sample.

Fig. 2. A mesh plot of \( r_1 \) relaxivity performance of Gd\textsuperscript{3+} DOTA\textsubscript{3} SNPs with variation of (i) ratio of two molecular building blocks, i.e., Ad-PAMAM and DOTA-CD-PEI, and (ii) loading of Gd\textsuperscript{3+} (24 data points). The peak value indicates the highest \( r_1 \) relaxivity (Gd\textsuperscript{3+} DOTA\textsubscript{3} SNPs with a ratio of 1:2 and at a Gd\textsuperscript{3+} concentration of 3.9 mM).

Fig. 3. (A) Dynamic light scattering (DLS) results and (B) TEM image show the narrow size distribution and spherical morphology of the resulting Gd\textsuperscript{3+} DOTA\textsubscript{3} SNPs (formulated with a ratio of 1:2 and at a Gd\textsuperscript{3+} concentration of 3.9 mM). (C) Stability studies of Gd\textsuperscript{3+} DOTA\textsubscript{3} SNPs before (left) and after 24 h incubation under different conditions: 400 times dilution in water (center) and 10% serum (right). All the sizes of Gd\textsuperscript{3+} DOTA\textsubscript{3} SNPs were characterized by TEM after 24 h incubation. (Each sample has at least 20 counts.)
2.8. Encapsulation efficiency of Gd\(^{3+}\)·DOTA<sub>3</sub>SNPs

The encapsulation efficiency is defined as the amount of Gd\(^{3+}\) within the SNPs divided by the total amount of Gd\(^{3+}\). 400 μL of Gd\(^{3+}\)·DOTA<sub>3</sub>SNPs prepared at various concentrations of Gd\(^{3+}\) (0.7, 1.4, 2.1, 3.5, 6.0, 6.7, 9.2, 11, 12, 14 and 17 mM) was added into a centrifugal filter at 10 K rpm for 30 min. After recovering filtrate containing free Gd\(^{3+}\) and supernatant containing encapsulated Gd\(^{3+}\), the quantity of Gd\(^{3+}\) in both solutions was quantified by ICP-MS. The encapsulation efficiencies of each resulting Gd\(^{3+}\)·DOTA<sub>3</sub>SNPs are summarized in Fig. S1.

2.9. Relaxation measurements

\(T_1\) relaxation rate measurements were performed on a Bruker Avance 600 MHz spectrometer (Bruker BioSpin Corp., Billerica, MA, USA) equipped with a narrow-bore (54 mm) 14.1 T magnet and a broadband probe at 295 K. Both inversion recovery (IR) and saturation recovery (SR) pulse sequences were acquired.

2.10. Phantom preparation and imaging

For the dilution phantom imaging study, 90 mm capillaries were filled with the diluted solution at concentrations ranging from 4.88 μM to 195 μM and placed together in an 8 mm NMR tube containing D₂O. Imaging was performed on a Bruker Avance 600 MHz spectrometer (Bruker BioSpin Corp., Billerica, MA, USA) equipped with a Micro 5 gradient system (maximal gradient strength of 100 G/cm in three orthogonal directions). A saturation recovery pulse sequence was used for phantom \(T_1\)-weighted imaging and the magnetization evolved 2.5 s before applying a spin echo pulse sequence to acquire the image.

2.11. In vivo imaging

In vivo imaging studies used 4–6 week-old female NOD CB17-Prkd<sup>+/-</sup> IcrCr1B1tww (NOD/SCID), weighing 25–30 g, obtained from BioLASCO, Taiwan. All animal procedures were in accordance with the regulations approved by the Institutional Animal Care and Utilization Committee at National Taiwan University. All the animal procedures were in accordance with the regulations approved by the Institutional Animal Care and Utilization Committee at National Taiwan University. All the MR data were acquired on a Varian INOVA 7-T NMR spectrometer (Varian Inc., CA, USA) with microimaging capability. The images were obtained using a microimaging probehead and a 30 mm I.D. quadrature birdcage imaging RF coil (Varian Inc., CA, USA) with self-shielded gradient systems and a maximum strength of 100 G/cm in each of the x-, y- and z-directions (Resonance Research Inc., Billerica, MA, USA). \(T_1\)-weighted images were acquired along the coronal plane using a multiple-slice spin echo pulse sequence with the following parameters: TR/TE = 50/12 ms, matrix = 256 × 128, FOV (field of view) = 51.2 × 25.6 mm, slice thickness = 0.25 mm, NT = 4. We acquired 2 slices during each TR and total number of slices = 96. The total acquisition time was about 25 min. The three-dimensional \(T_1\)-weighted images were acquired before injection of Gd\(^{3+}\)·DOTA<sub>3</sub>SNPs and at the following times after injecting 20 μL of Gd\(^{3+}\)·DOTA<sub>3</sub>SNPs in the right foot pad of the NOD/SCID mouse: 40–65 min, 100–125 min, 160–185 min, 220–245 min, 280–305 min, and 340–365 min. Additionally, we acquired images 12 h and 24 h after injecting Gd\(^{3+}\)·DOTA<sub>3</sub>SNPs. No gross side effects were observed during or after injection of Gd\(^{3+}\)·DOTA<sub>3</sub>SNPs. For quantitative analysis of acquired images, we take the average signal in a 11 × 9 × 8 voxel volume containing the lymph nodes indicated by the circles, the region of interest (ROI).

3. Results and discussion

The three molecular building blocks, Gd\(^{3+}\)·DOTA-CD-PEI, Ad-PAMAM, and Ad-PEG (Fig. 1), were prepared and characterized according to literature [24]. By systematically changing (i) the concentration of encapsulated Gd\(^{3+}\) (0.32, 0.63, 1.61, 3.22, 3.90 and 6.44 mM) and (ii) the Ad-PAMAM/DOTA-CD-PEI ratios (1:1, 2:3, 1:2 and 1:3), a small library composed of 24 different formulations of Gd\(^{3+}\)·DOTA<sub>3</sub>SNPs (Fig. 2) was prepared. The size and cross-linking degree of the hydrogel core are dependent on the Ad-PAMAM/DOTA-CD-PEI ratio. The 24 different formulations of Gd\(^{3+}\)·DOTA<sub>3</sub>SNPs result from mixing Gd\(^{3+}\)·DOTA-CD-PEI, prepared at one of six Gd\(^{3+}\) concentrations, with solutions of Ad-PAMAM/Ad-PEG prepared at one of four Ad-PAMAM concentrations. The dynamic nature of the supramolecular synthetic approach leads to 80–95% of Gd\(^{3+}\) encapsulated into Gd\(^{3+}\)·DOTA<sub>3</sub>SNPs, which was quantified by ICP-MS (see
Supporting information, Fig. S1). A broad spectrum of \( r_1 \) emerges from the resulting \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs library with \( r_1 \) ranging from 4.19 s\(^{-1}\) mM\(^{-1}\) to 17.3 s\(^{-1}\) mM\(^{-1}\). Measurements were performed in water on a 600 MHz NMR spectrometer and are presented in a mesh plot in Fig. 2. The results highlight an optimal synthetic formulation of \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs (1:2 M ratio and 3.9 mM of \( \text{Gd}^{3+} \)) with a relaxivity of 17.3 s\(^{-1}\) mM\(^{-1}\) (proposed attribution of relaxation enhancement described in Supporting information, Fig. S4). We do not measure a difference in \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs relaxation between 300 and 600 MHz (results not shown). This is significantly higher than clinical \( \text{Gd}^{3+} \) chelated complexes, i.e., \( \text{Gd}^{3+} \)-DTPA, which has \( r_1 \) ~4.0 s\(^{-1}\) mM\(^{-1}\) at 300 MHz and 600 MHz. We characterize the resulting \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs by measuring the hydrodynamic size of \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs with DLS (Fig. 3A) and the results are consistent with TEM images. As shown in Fig. 3B, the TEM images indicate that \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs have an average diameter of 103 \( \pm \) 10 nm with a spherical morphology and narrow size distribution. The hydrodynamic size of \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs (124 nm) measured by DLS analysis was larger than the value obtained from TEM, probably due to the swelling of the PEG shell layer on the \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs in water. The zeta potential of the resulting optimal \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs is 22.5 \( \pm \) 1.4 mV. The stability tests of \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs were carried out under two conditions: (i) 400 times dilution in water and (ii) 10% serum. The solutions were prepared from \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs with 3.9 mM of \( \text{Gd}^{3+} \) and then incubated under the conditions for 24 h. The sizes of both solutions were characterized by TEM and summarized in Fig. 3C. The original size of \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs is 103 \( \pm \) 10 nm (Fig. 3C, left). The size after 400 times dilution is 112 \( \pm \) 15 nm (Fig. 3C, center) and in 10% serum is 101 \( \pm \) 9 nm (Fig. 3C, right). There are no distinct changes in the average sizes of \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs after dilution and in 10% serum; therefore, we conclude that the \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs remain stable under these conditions.

To test feasibility of \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs as a sensitive MRI CA, we performed a series dilution study of \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs with deionized (DI) water to establish the minimum \( \text{Gd}^{3+} \) concentration detectable by MRI. Fig. 4 shows \( T_1 \)-weighted imaging results of the series dilution for \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs solution and \( \text{Gd}^{3+} \)-DTPA (Fig. 4A) at seven concentrations (from 195 to 4.88 \( \mu \)M) and Fig. 4B plots the C/N ratios as a function of \( \text{Gd}^{3+} \) concentration for \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs (circles) and \( \text{Gd}^{3+} \)-DTPA (squares). The \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs and \( \text{Gd}^{3+} \)-DTPA phantoms were imaged separately (note different scale bars) and the C/N ratio between \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs and DI water are higher than the C/N ratio between \( \text{Gd}^{3+} \)-DTPA and DI water at all concentrations. The results indicate that the significant sensitivity improvement and relaxivity enhancement of \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs compared to \( \text{Gd}^{3+} \)-DTPA is maintained after diluting the CA solutions down to \( \mu \)M concentrations of \( \text{Gd}^{3+} \).

The lymphatic system is a common route for metastatic spread of cancer [28–30]. Dynamic imaging of lymphatic drainage has been regarded as a powerful diagnostic protocol for monitoring cancer metastasis. We attempted to explore the use of \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs as a new type of NPs for dynamic imaging of lymphatic drainage compared with conventional \( \text{Gd}^{3+} \) chelated complexes. Fig. 5A shows 3D maximum intensity projection (MIP) [31] \( T_1 \)-weighted in vivo imaging results before and after injecting \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs into NOD/SCID mice (experimental details in Supporting information). The yellow circles contain the right brachial lymph node of the mouse.

**Fig. 5.** (A) Three-dimensional (3D) \( T_1 \)-weighted MR images show dynamic lymphatic drainage of \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs. The yellow circle contains the right brachial lymph node. The five images were acquired before injecting and at four times after injecting 20 \( \mu \)l of \( \text{Gd}^{3+} \)-\( \text{DOTA} \)-SNPs (formulate with a ratio of 1:2 and a \( \text{Gd}^{3+} \) concentration of 3.9 mM) as labeled. (B) Average voxel signal intensity plotted as a function of image acquisition time (0 h represents data acquired before injecting the MRI CA). (C) \( \text{Gd}^{3+} \) concentration from ICP-MS measurements on lymph node tissue removed 30 min and 2.5 h after injecting the MRI CA. Dashed line represents the baseline measurement on untreated brachial lymph node tissue. Note the log scale for the \( \text{Gd}^{3+} \) concentration axis.
We injected 20 µL of a 3.9 mM MRI CA solution into the front right foot pad of a NOD/SCID mouse and monitored the changes with MRI. The draining of the lymph nodes was clearly visualized with the Gd³⁺-DOTA-C SNPs. The average voxel signal intensity increases in the right brachial lymph node after injecting Gd³⁺-DOTA-C SNPs but not after injecting Gd³⁺-DTPA (Fig. 5B and also see Supporting information Fig. S2). Meanwhile, the average signal intensity in the left brachial lymph node remains around 20% less than the right lymph node (see Supporting information, Fig. S3). After 12 h, the average signal intensity in both lymph nodes decreases close to the values before injecting the Gd³⁺-DOTA-C SNPs. To support detection with MRI of Gd³⁺-DOTA-C SNPs drainage in the brachial lymph node, we used ICP-MS analysis on ex vivo tissue removed at two time points after injecting MRI CA, i.e., Gd³⁺-DOTA-C SNPs and Gd³⁺-DTPA, into the front foot pads of the mice following the same procedure used for in vivo imaging. We removed the brachial lymph nodes of mice after 30 min and 2.5 h. The brachial lymph nodes were dissected and underwent ICP-MS measurement. The ICP-MS analysis revealed that Gd³⁺ concentration decreased by approximately 6% after 2.5 h. As a comparison, the Gd³⁺ concentration from mice treated with Gd³⁺-DTPA was negligible and close to the baseline measurement on untreated brachial lymph node tissue. The results show that the ICP-MS measurements are in good agreement and validate the T₁-weighted MRI results (experimental details of ICP-MS analysis are in Supporting Information).

4. Conclusion

We have successfully demonstrated the systematic synthesis of a small library of Gd³⁺-DOTA-C SNPs by a supramolecular self-assembly approach. The results highlight an optimal synthetic formulation of Gd³⁺-DOTA-C SNPs with an r₁ of 17.3 s M⁻¹ mm⁻¹, which is ca. 4-fold higher than the Gd³⁺-chelated complexes at high field strengths. In addition, the resulting Gd³⁺-DOTA-C SNPs were detected by T₁-weighted imaging at a concentration of 4.88 µM Gd³⁺ with a contrast-to-noise ratio (C/N ratio) of 9.46, ca. 3.6 times greater than the C/N ratio for Gd³⁺-DTPA at the same concentration, signaling a higher sensitivity than conventional MRI CAs. Moreover, the dynamic lymph node drainage MRI demonstrated the applicability of Gd³⁺-DOTA-C SNPs in vivo.

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Appendix

Figures with essential colour discrimination. Figs. 1–3 and 5 in this article, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.11.043.

Appendix. Supporting information

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2010.11.043.