Use of unnatural amino acids for design of novel organomodified clays as components of nanocomposite biomaterials

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Sodium montmorillonite (Na-MMT) clay was modified with three different unnatural amino acids in order to design intercalated clay structures that may be used for bone biomaterials applications. Prior work on polymer–clay nanocomposites (PCNs) has indicated the effect of the appropriate choice of modifiers on enhancing properties of PCNs. Our X-ray diffraction results indicate an increase in the $d$-spacing of Na-MMT clay after it was modified with the three unnatural amino acids. Transmission Fourier transform infrared spectroscopy experiments were carried out on the unmodified and modified MMT clay samples to study the molecular interactions between the amino acids used as modifiers and the Na-MMT clay. Cell culture experiments showed that the Na-MMT clay modified with the three amino acids was biocompatible as were the modified clay-incorporated films of chitosan/polygalacturonic acid/hydroxyapatite.

Keywords: Na-montmorillonite; ($\pm$)-2-aminopimelic acid; 5-aminovaleric acid; DL-2-aminocaprylic acid; $d$-spacing; biocompatibility

1. Introduction

Tissue engineering is ‘an interdisciplinary field of research that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain or improve tissue function’ (Langer & Vacanti 1993). The main focus of tissue engineering is the development of materials with adequate mechanical properties that favour tissue formation, differentiation and regeneration. In this regard, there has been a continuous thrust in recent years for developing composite systems using polymers and fillers in the micrometre–nanometre range that can satisfy the requirements such as biocompatibility, biodegradability, surface properties and mechanical properties needed for materials used for tissue engineering.

In addition, extensive research on polymer–clay nanocomposites (PCNs) since 1990 when the Toyota research group (Okada et al. 1990) reported significant findings for nylon–clay nanocomposites has paved the way for developing advanced composite materials based on polymer–clay composite systems. PCNs

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are composites that consist of clay particles having at least one of their dimensions in the nanometre range dispersed in a polymer matrix (Alexandre & Dubois 2000). PCNs show significant improvement in properties when compared with neat polymer and polymers containing micron-sized fillers. Several studies have reported that PCNs show improvement in mechanical properties (Okada et al. 1990; Giannelis 1996; Chen et al. 2002; Maity et al. 2002; Lim et al. 2003; Ma et al. 2003; Park et al. 2003; Pramanik et al. 2003; Pramoda et al. 2003), a decrease in gas permeability (Yano et al. 1993; Messersmith & Giannelis 1995; Bharadwaj 2001; Xu et al. 2001) and flammability (Gilman et al. 1997, 2000; Gilman 1999) and also affect the biodegradability of biodegradable polymers (Ray et al. 2002). There has also been considerable interest in modelling and in the simulation and development of new theories for PCNs. A recent simulation study has reported the development of an altered phase concept for PCNs (Sikdar et al. 2008a–c). According to this study, molecular-level interactions between polymer and clay in PCNs create an ‘altered polymer phase’, with different elastic properties from the neat polymer. This ‘altered polymeric phase’ was responsible for the increase in the mechanical properties of PCNs. In another simulation study related to PCNs, it was found that the properties of the clay and polymer and the interactions at the interfaces between them change the properties of both the polymer and the clay, which played an important role in the mechanical behaviour of PCNs (Sikdar et al. 2008a–c).

Nanosized montmorillonite (MMT) clay has been an important constituent of PCNs and is responsible for their significantly improved properties. Sodium MMT (Na-MMT) is a layered silicate from the family of 2:1 phyllosilicates (Alexandre & Dubois 2000; Ray et al. 2003; Pavlidou & Papaspyrides 2008). There is one octahedral alumina sheet between two silica tetrahedral sheets in each layer of MMT (figure 1). The thickness of each layer is in the nanometre range, and its lateral dimension is in the micrometre range. Isomorphic substitutions take place in the layers of Na-MMT that generate negative charge, which is balanced by
Organomodified nanoclays as biomaterials

the exchangeable cations such as sodium, calcium and magnesium present in the interlayer spacing. Pure MMT clay is hydrophilic and can be made organophilic by exchanging the cations present in the interlayer with cationic surfactants such as alkylammonium or alkylphosphonium ions. The cationic surfactants may also increase the interlayer spacing and thus facilitate the intercalation of the polymeric species in the interlayer (Alexandre & Dubois 2000; Ray et al. 2003; Paiva et al. 2008; Pavlidou & Papaspyrides 2008).

MMT has medicinal properties because it has the ability to adsorb dietary toxins, bacterial toxins associated with gastrointestinal disturbance, hydrogen ions in acidosis and metabolic toxins; possesses mucoadhesive capability to cross the gastrointestinal barrier; and also has drug-carrying capacity (Forni et al. 1989; Lee & Fu 2003; Dong & Feng 2005; Lee et al. 2006; Viseras et al. 2007). It is used as an inactive substance for carrying the active ingredients of a medication and is also used as an active substance for pharmaceutical applications (Wang et al. 2008). Several studies involving the use of MMT clay for drug delivery and drug release have been reported (Takahashi et al. 2005; Rieux et al. 2006; Bingfeg et al. 2008; Wang et al. 2008; Depan et al. 2009).

Although MMT clay has medicinal properties and several studies involving the use of MMT clay for drug release and drug delivery applications have been carried out to date, a limited number of studies related to the utilization of MMT for structural application in biomaterials have been reported. Few studies related to the use of chitosan and MMT have been reported (Darder et al. 2005; Wang et al. 2005, 2006; Gunister et al. 2007; Wang et al. 2007). Xu et al. (2006) prepared chitosan–MMT nanocomposite films with an improved tensile strength. Wang et al. (2005) prepared exfoliated–intercalated chitosan–MMT nanocomposites and found that the presence of MMT clay improved the thermal properties and increased the hardness and elastic modulus of the composites. Lin et al. (2005) developed chitosan–clay nanocomposites that showed an improvement in tensile strength and a decrease in in vitro degradation. In our previous work, we

Figure 2. Molecular structure of (a) (±)-2-aminopimelic acid, (b) 5-aminovaleric acid and (c) DL-2-aminocaprylic acid.
K. S. Katti et al.
synthesized a novel chitosan–MMT–hydroxyapatite (HAP) nanocomposite that showed a significant increase in nanomechanical property when compared with the chitosan–HAP and chitosan–MMT composites (Katti et al. 2008). Also, poly-(ε-caprolactone)–MMT nanocomposites were prepared by electrospinning, which exhibited improved stiffness when compared with neat poly-(ε-caprolactone) without any significant decrease in the ductility of the polymer (Marras et al. 2008). Zheng et al. (2007) developed a gelatin–MMT–chitosan scaffold that showed an improvement in the mechanical properties owing to the addition of MMT and also found that the degradation rate was affected by the MMT clay content.

Modification of clays plays an important role in the preparation of nanocomposites since it can affect the final properties of the nanocomposites. The extent of intercalation of the polymer in the interlayer spacing of clay is affected by the interaction between the polymer and the functional groups of the modifier used for clay modification. Our prior simulation study in using amino acids to intercalate clays has indicated that clays may be intercalated with amino acids (Katti et al. 2005). In addition, our work involving PCNs indicated the role of chain length and functionality of a modifier on the ability to intercalate the clay (Sikdar et al. 2009). Hence, we chose amino acids with longer chains as modifiers in this work. Molecular dynamics simulation studies related to PCNs have shown the importance of the effect of interactions among polymer, organic modifiers and clay on the crystallinity and nanomechanical properties of PCNs (Sikdar et al. 2008a–c). Also, we found that organic modifiers affect the crystallinity and nanomechanical properties of the prepared PCNs (Sikdar et al. 2007). Therefore, it is essential to use an appropriate modifier to obtain the required properties in the composites. In order to use MMT clay for structural application in biomaterials, it is necessary to increase its interlayer spacing and to simultaneously maintain or improve its biocompatibility. Unnatural amino acids possess the potential to be used as modifiers, which can increase the interlayer spacing of clay and also maintain or improve its biocompatibility. These are the ‘non-genetically coded amino acids’ (Ma 2003) that are either natural or synthetic and are gaining significance in drug research. They find use as chiral building blocks, molecular scaffolds in constructing combinatorial libraries and molecular probes for the better understanding of biological systems. (Dougherty 2000; Ma 2003). In this work, three different unnatural amino acids (figure 2) were used for modifying the MMT clay. X-ray diffraction (XRD) was used to study the intercalation of amino acids in the MMT clay. Clay interactions with water, fluids, modifiers and polymers have been investigated extensively in our group previously using Fourier transform infrared spectroscopy (FTIR) (Katti & Katti 2006; Amarasinghe et al. 2008, 2009). The modified clays were characterized by FTIR, and the biocompatibility of the modified MMT clay was assessed through cell culture experiments.

2. Materials

Na-MMT (Swy-2, Crook County, WY, USA) with a cationic exchange capacity of 76.4 mequiv per 100 g was obtained from the Clay Minerals Repository at the University of Missouri, Columbia, MO, USA. The three amino acids,
namely (±)-2-aminopimelic acid, 5-aminovaleric acid and DL-2-aminocaprylic acid, were obtained from Sigma-Aldrich. Silver nitrate (0.1N) was purchased from Anachemia Chemicals, and 1 N hydrochloric acid (HCl) was purchased from VWR International. Chitosan (more than 85% deacetylated) and polygalacturonic acid (PgA) (approx. 95% enzymatic) were obtained from Sigma-Aldrich. For conducting cell culture experiments, HyQ Dulbecco’s modified Eagle’s medium (DMEM)-12 (1 : 1) from Hyclone and G418 solution (antibiotic) from JR Scientific were used. Osteoblast cells (cell line number CRL-11372) and foetal bovine serum (FBS) were purchased from American Type Culture Collection.

3. Experiments

(a) Preparation of modified MMT clays

Na-MMT was ground into fine powder and then screened through a no. 325 sieve (45 μm). About 5 g of this fine and sieved sodium MMT was placed in an oven for heating for 12 h at 60°C. Thus, the amino acid-processed sodium MMT was then dispersed into 400 ml of deionized (DI) water pre-heated to 60°C. In another beaker, 1.9 g of amino acid was added to 100 ml of DI water pre-heated to 60°C. Further, the pH of the amino acid solution was maintained at 1.8 by addition of 0.1 N HCl. The amino acid solution was then added to the MMT clay suspension, and the resulting solution was stirred vigorously for 1 h at a pH of 1.8 and at 60°C. The modified MMT was separated by centrifuging and further washed several times with DI water until the Cl$^-$ ions were removed completely from the clay. The filtrate obtained after centrifuging was titrated with 0.1N silver nitrate. The titration procedure was repeated until no white precipitate was formed, which indicated the complete removal of chloride ions. Finally, the modified clay was placed in an oven for 24 h at 70°C. The clay was then ground and passed through a no. 325 sieve (45 μm). The aforementioned method was used for the preparation of modified clays with the three amino acids used in this study.

(b) Preparation of chitosan–PgA–HAP–MMT composite films

Chitosan (Chi) and PgA solutions were prepared separately by dissolving 1 g each of chitosan and PgA in 100 ml of DI water. The fabrication of ChiPgAHAP composites has been described previously (Verma et al. 2008a, b, 2009). Acetic acid was used for dissolving chitosan in DI water, and diluted sodium hydroxide solution was used for dissolving PgA in DI water. Chitosan solution was added dropwise to PgA solution, and the resulting mixture was sonicated to obtain ChiPgA polyelectrolyte complex solution. HAP was prepared by using the wet precipitation method (Katti et al. 2006). HAP (20 wt%) and MMT clay modified with 5-aminovaleric acid (10 wt%) were dispersed in DI water by sonication separately and added to the ChiPgA polyelectrolyte complex solution. The resulting mixture was sonicated for proper mixing of HAP and modified clay. Films were prepared by adding the ChiPgAHAPMMT solution (1 : 10 dilution) to tissue culture polystyrene Petri dishes and subsequent evaporation under atmospheric conditions. These films were used for determining biocompatibility through cell culture.

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(c) XRD characterization

An X-ray diffractometer (Philips X’pert, Almelo, The Netherlands) equipped with a secondary monochromator and Cu-tube using CuKα radiation of wavelength 1.54056 Å was used to obtain XRD data on powdered clay samples. The d-spacing of the clay structure was calculated from the XRD data using Bragg’s diffraction law. A scan range of 2θ = 2–30° and the scan rate of 2° min⁻¹ were used. Powdered MMT clay and modified clay samples were placed in an aluminium mount prior to performing the XRD scan.

(d) FTIR characterization

FTIR experiments were conducted using a ThermoNicolet, Nexus, 870 spectrometer with a KBr beam splitter in the spectral range of 4000–400 cm⁻¹ at a spectral resolution of 4 cm⁻¹ using a mirror velocity of 0.158 cm s⁻¹. FTIR experiments were performed by using the transmission accessory. Samples for FTIR experiments in the form of pellets were prepared from the mixture of KBr and powdered clay. The samples were placed in the universal sample holder for conducting the FTIR experiments.

(e) Cell culture

Powdered MMT and modified clays (1.5 mg each) were placed in tissue culture polystyrene Petri dishes and then sterilized by placing these Petri dishes for 2.5 h under ultraviolet (UV) light. Tissue culture polystyrene Petri dishes without clay samples were also sterilized under UV light. About 40 000 osteoblast cells were then seeded into these Petri dishes, which have a growth area of 19.5 cm². Cells were allowed to grow in the Petri dishes in the presence of 1.5 ml of cell culture medium, which consisted of 90 per cent HyQ DMEM-12 (1:1), 10 per cent FBS and 0.6 per cent G418 solution (antibiotic) for each sample. The Petri dishes containing the samples were then placed in an incubator at 37°C and under 5 per cent CO₂. The growth of the cells in Petri dishes containing the clays was investigated by taking photographs after 48 h of seeding the cells on the powdered clay samples by using an inverted microscope.

For determining the biocompatibility of the ChiPgAHAPMMT films, osteoblast cells were seeded on these films in the Petri dishes. Osteoblast cells at 95 per cent confluence were obtained from T-flasks. An aliquot of 0.5 ml cell solution was added to the Petri dishes containing the films along with 1.5 ml of cell culture medium. The behaviour of the osteoblast cells on the films was observed using an inverted microscope.

4. Results and discussion

(a) XRD results

The XRD patterns of MMT clay and MMT clay modified with the three amino acids used in this study are shown in figure 3a–c. The peak corresponding to the d₀₀₁ plane of pure MMT clay is observed at 2θ = 8.842°. The d₀₀₁ spacing
Organomodified nanoclays as biomaterials

at this value of $2\theta$ is 9.992 Å. In the case of MMT clay modified with (±)-2-aminopimelic acid, the peak appears at $2\theta = 7.943^\circ$, which corresponds to a $d_{001}$ spacing of 11.121 Å. For MMT clay modified with 5-aminovaleric acid, the peak appears at $2\theta = 6.912^\circ$, which corresponds to a $d_{001}$ spacing of 12.771 Å. The peak for MMT clay modified with DL-2-aminocaprylic acid is seen at $2\theta = 6.757^\circ$, which corresponds to a $d_{001}$ spacing of 13.070 Å. Thus, the $d_{001}$ spacing of MMT clay shows an increase after modification with amino acids, which indicates the formation of an intercalated structure. Figure 4 shows the comparative increase in the interlayer spacing of MMT clay after modification with the amino acids. It is observed that the increase in the interlayer spacing is the highest in the case of MMT clay modified with DL-2-aminocaprylic acid. The difference in the extent of increase in interlayer spacing may be due to differences in the positions or number of the −COOH and −NH$_2$ groups in the three amino acids. The molecule DL-2-aminocaprylic acid has one extra −CH$_2$ group besides the methyl −CH$_3$ group when compared with (±)-2-aminopimelic acid and 5-aminovaleric acid. This suggests that the increase in the $d$-spacing of MMT clay can be affected by the length of the hydrocarbon chain and the position of the functional groups in the intercalating molecule.

Figure 3. (a) XRD of MMT and MMT modified with (±)-2-aminopimelic acid, (b) XRD pattern of MMT and MMT modified with 5-aminovaleric acid and (c) XRD pattern of MMT and clay modified with DL-2-aminocaprylic acid.
Figure 4. Comparative increase in interlayer spacing of MMT clay after modification with amino acids.

(b) FTIR results of (±)-2-aminopimelic acid, 5-aminovaleric acid, DL-2-aminocaprylic acid, MMT clay and MMT clay modified with (±)-2-aminopimelic acid, 5-aminovaleric acid and DL-2-aminocaprylic acid

Figure 5a,b shows the FTIR spectra for the three amino acids within the 4000–400 and 2000–1260 cm\(^{-1}\) regions, respectively. Table 1 shows the band assignments seen in this region. The bands in the 1680–1682 cm\(^{-1}\) region are due to the intramolecular hydrogen bonding owing to the carboxylic groups in the three amino acids. Figure 6a–f shows the FTIR spectra for MMT clay and MMT clay modified with the three amino acids. The corresponding band assignments are given in table 2. Figure 6a shows the FTIR spectra of the modified clays within the 4000–400 cm\(^{-1}\) range. Figure 6b shows the spectra for the clays modified with the three amino acids within the 3800–2100 cm\(^{-1}\) range. The clays modified with amino acids show a shift in the band position corresponding to the structural OH group when compared with pure MMT clay. In figure 6c, it is observed that there are differences in the FTIR spectra of the modified clays and the pure MMT clay. In the case of the clays modified with the amino acids, bands are seen at 1733, 1716 and 1731 cm\(^{-1}\), which indicate the presence of the amino acids in the modified clays. These bands are attributed to the C=O stretching bands of the carboxylic group in the amino acid. There is a shift in the position of the C=O stretching band to a lower frequency by 15 cm\(^{-1}\) in the case of MMT clay modified with 5-aminovaleric acid, which suggests hydrogen bonding interactions between 5-aminovaleric acid and MMT clay. Figure 6c also shows that there is a shift in the band positions associated with H−O−H deformation in the case of clays modified with the three amino acids. Also, the bands in the 1681–1680 cm\(^{-1}\) region observed in the case of the three amino acids are absent in the case of all the three modified clays, which indicates that the intramolecular hydrogen bonding of the three amino acids is broken due to the possible interaction of the amino acids with
Figure 5. FTIR spectra of the three amino acids within the (a) 4000–400 cm$^{-1}$ range and (b) 2000–1260 cm$^{-1}$ range.

Table 1. Band assignment for amino acids.

<table>
<thead>
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<th>band position (cm$^{-1}$)</th>
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<tr>
<td>3777, 3754, 3752</td>
<td>O–H and N–H stretching</td>
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<tr>
<td>3723, 3713, 3706</td>
<td>O–H and N–H stretching</td>
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<tr>
<td>2369, 2338, 2319</td>
<td>N–H$^+$ stretching</td>
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<tr>
<td>1681, 1680</td>
<td>C=O stretching vibration (intramolecular hydrogen bonding in carboxylic acids)</td>
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<tr>
<td>1732, 1731, 1728</td>
<td>C=O stretching</td>
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<tr>
<td>1584, 1588</td>
<td>R–COO$^-$ symmetric stretching</td>
</tr>
<tr>
<td>1534, 1504, 1489, 1468</td>
<td>N–H vibrations</td>
</tr>
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</table>
Figure 6. FTIR spectra of MMT clay modified with amino acids within the (a) 4000–400 cm$^{-1}$ range, (b) 3800–2100 cm$^{-1}$ range and (c) 2040–1535 cm$^{-1}$ range. (d) Second derivative FTIR spectra of MMT clay modified with amino acids within the 1655–1595 cm$^{-1}$ range. (e) FTIR spectra of MMT clay modified with amino acids within the 1394–835 cm$^{-1}$ range. (f) Second derivative FTIR spectra of MMT clay modified with amino acids in the 1394–835 cm$^{-1}$ range.
the clay. Figure 6d shows the second derivative spectrum of the modified clays in the 1645–1600 cm$^{-1}$ region. It is seen that, in the case of clays modified with 5-aminovaleric acid and DL-2-aminocaprylic acid, there is an overlap of two bands in this region. The bands near the 1616 cm$^{-1}$ region in the case of clays modified with 5-aminovaleric acid and DL-2-aminocaprylic acid may be due to the N–H deformation vibrations that arise owing to the presence of these acids in the MMT clay. The absence of N–H deformation near the 1616 cm$^{-1}$ region in the case of clay modified with (±)-2-aminopimelic acid may be attributed to the presence of two carboxylic groups, which may mask the effect of the presence of the amine group of (±)-2-aminopimelic acid. Figure 6e shows the FTIR spectra of modified and unmodified clays within the 1394–835 cm$^{-1}$ region. The corresponding second derivative spectrum for this region is given in figure 6f. The band assignments for this region are given in table 2. There are shifts in the Si–O stretching vibrations in the case of clays modified with the amino acids. These shifts may be attributed to the interaction between the protonated amine group and the surface oxygen of silica tetrahedral. Also, the band at the 1210 cm$^{-1}$ position observed in the
Table 2. Band assignment for MMT clay and MMT clay modified with amino acids.

<table>
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<td>3646</td>
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<td>1638</td>
<td>MMT clay</td>
<td>H–O–H deformation</td>
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<td>MMT clay modified with (±)-2-aminopimelic acid</td>
<td>Si–O out-of-plane stretching</td>
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<td>MMT clay modified with 5-aminovaleric acid</td>
<td>Si–O stretching</td>
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<td>910</td>
<td>MMT clay modified with DL-2-aminocaprylic acid</td>
<td>Al–OH deformation</td>
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<td>879</td>
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<td>Al–FeOH deformation</td>
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The case of pure MMT clay seems to be absent in the case of clays modified with 5-aminovaleric acid and DL-2-aminocaprylic acid. This may be an implication that the crystal structure of MMT clay has changed due to intercalation of amino acids in MMT clay. Therefore, the unpolarized infrared beam is unable to cause Si–O out-of-plane stretching vibration in the case of the modified clays.

(c) Cell culture results

The number of osteoblast cells grown in the presence of MMT clay and MMT clay modified with amino acids was counted manually from the images taken with an inverted microscope after 48 hours of seeding the osteoblast cells. The cell density (cells mm$^{-2}$) for the modified clay samples was calculated and plotted as shown in figure 7a,b. The images in figure 8 show that the osteoblast cells grow in the presence of MMT clay and MMT clay modified with the amino acids. The growth of osteoblast cells is comparatively high in the case of MMT clay.
Organomodified nanoclays as biomaterials

Figure 7. Comparative cell density data after 48 h for (a) MMT clay and MMT clay modified with the three amino acids and (b) MMT clay, MMT clay modified with the three amino acids and tissue culture polystyrene.

modified with 5-aminovaleric acid and comparatively low in the case of MMT clay modified with DL-2-aminocaprylic acid. Figure 9 shows that osteoblast cells attach to ChiPgAHAPMMT films. This indicates that the MMT clay modified with 5-aminovaleric acid has potential for biomaterial applications.

5. Conclusions

MMT clay was intercalated with three unnatural amino acids: (±)-2-aminopimelic acid, 5-aminovaleric acid and DL-2-aminocaprylic acid. The intercalation of the clays by all the three amino acids was confirmed by XRD. Transmission FTIR experiments of clays modified with the three amino acids showed shifts in band positions of C=O vibration that were indicative of significant molecular interactions between the amino acids and MMT clay. Our prior work on molecular
Figure 8. Inverted microscopic images of osteoblast cells cultured in (a) tissue culture polystyrene Petri dishes, (b) MMT clay on tissue culture polystyrene Petri dishes and (c) MMT clay with aminovaleric, (d) MMT with amino caprylic and (e) MMT with aminopimelic acid on tissue culture polystyrene Petri dishes. Scale bars, (a–e) 500 μm.

Figure 9. Inverted microscopic image of osteoblast cells seeded on ChiPgAHAPMMT films after 4 days. Scale bar, 500 μm.
dynamics simulations of interactions between constituents of PCNs indicates that significant non-bonded interactions, such as observed here between clay and modifier, have a large impact on the final mechanical response of the nanocomposite. Cell culture experiments showed that all the three modified clays were biocompatible. Thus, the MMT clays modified with the three amino acids used in this study show potential for structural application in biomaterials, as indicated by the cell behaviour on the ChiPgAHAPMMT films prepared by using the modified clay.

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Organomodified nanoclays as biomaterials


Phil. Trans. R. Soc. A (2010)


