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Clay nanoparticles for regenerative medicine and biomaterial design: a review of clay bioactivity

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Abstract: Clay nanoparticles, composites and hydrogels are emerging as a new class of biomaterial with exciting potential for tissue engineering and regenerative medicine applications. Clay particles have been extensively explored in polymeric nanocomposites for self-assembly and enhanced mechanical properties as well as for their potential as drug delivery modifiers. In recent years, a cluster of studies have explored cellular interactions with clay nanoparticles alone or in combination with polymeric matrices. These pioneering studies have suggested new and unforeseen utility for certain clays as bioactive additives able to enhance cellular functions including adhesion, proliferation and differentiation, most notably for osteogenesis. This review examines the recent literature describing the potential effects of clay-based nanomaterials on cell function and examines the potential role of key clay physicochemical properties in influencing such interactions and their exciting possibilities for regenerative medicine.

1. Introduction

Recent studies have shed new light on the potential of clay nanoparticles and composites for biomaterial design and regenerative medicine[1–3]. Clay nanoparticles are biocompatible at doses significantly higher than most other nanomaterials [4,5] and their degradation products are non-toxic, absorbable and of relevance to osteogenic cell function[6,7,8]. Furthermore, several studies have convincingly demonstrated direct, beneficial, concentration-dependent effects of clay nanoparticles on cellular adhesion, proliferation and differentiation [4–6,9–12]. These new observations combined with the well-established utility of clay nanoparticles to impart attractive mechanical or rheological properties to polymeric hydrogels and scaffolds [9–12,13, 14], and the opportunities afforded by their classic use as drug delivery modifiers[15,16], suggest the striking potential of clays for the creation and development of new bioactive scaffolds.

Clay minerals, also called sheet-silicates or phyllosilicates, are a family of inorganic layered nanomaterials classically defined as “minerals which impart plasticity to clay and which harden upon drying or firing”[17]. Based on archaeological and written records, clays have played an important role in medicine from the dawn of mankind, ranging from oral ingestion for therapeutic purposes (geophagy) to wound healing and haemorrhage inhibition[18,19]. Clays are still widely applied as active ingredients in pharmaceutical formulations, typically administered either orally as antacids, gastrointestinal protectors, and anti-diarrheic or topically as dermatological protectors and anti-inflammatories[20]. Clays also play an important role in pharmaceutical preparations as excipients functioning as disintegrants, diluents and binders, emulsifying, thickening and anticaking agents, flavour correctors and delivery modifiers of active agents [21,22].

Extensive research has been undertaken to investigate the role of clay minerals in drug/gene delivery and in the development of polymer-clay nanocomposites (PCNs). This interest is due

to the high retention capacities, swelling and rheological properties of clays and their affinity for interaction with biopolymers (either through exfoliation or intercalation). For instance, clay minerals can act as transport vehicles/carriers for the efficient delivery of therapeutic molecules (drugs and genes) by modifying the rate and/or time of release, increasing the stability of the drug or improving the dissolution profile of a drug[23]. Furthermore, the incorporation of a small percentage of clay nanoplatelets (dispersed phase) into a polymeric network (continuous phase) can improve the polymer's mechanical properties, swelling capacity, film-forming capability, rheological properties and bioadhesion without losing the inherent processability of the matrix[24].

The application of clay nanoparticles in drug/gene delivery, polymer clay nanocomposites and, more recently, regenerative medicine has been attributed to their unique physicochemical properties including particle size and shape, specific surface area, density of charge and structural and exchanged cations. These properties are dependent on the clay mineral type and crystal structure. Understanding how these clay structural/compositional parameters influence stem cell function will be important for exploring the fundamental mechanisms underlying clay bioactivity, and in the ability to exploit these properties in the development of 3D matrices, niche environments or delivery scaffolds for regenerative medicine.

In this review, we will introduce key aspects of clay chemistry with a specific focus on biomaterial design for regenerative medicine. We highlight recent advances in the development of clay-based biomaterials and discuss the evidence for the biocompatibility of clays. Finally, we explore the various mechanisms of clay bioactivity including modulation of cell adhesion, protein localisation, and biomineralisation as well as the possibility of clay nanoparticles to directly affect the osteogenic differentiation of skeletal populations.

2. An overview of clay chemistry

Broadly, clay minerals are structured of two principal units: tetrahedral (T) and octahedral (O) sheets[25,26]. Each tetrahedron consists of a central cation (mostly Si^{4+}) coordinated to four O^{2-} anions, and linked to adjacent tetrahedra through three shared oxygens on the corners (basal oxygens O_b) forming an infinite 2D hexagonal mesh (Figure 1). The fourth O^{2-} (apical oxygen O_a) remains unshared lying perpendicular to the tetrahedral sheet, and is the main site of interaction with the octahedral sheet. Each octahedron consists of a metal cation (M^{n+}) coordinated to six O^{2-} and/or OH^- anions. Adjacent octahedra are linked to each other by sharing edges (two O^{2-} or OH^-), forming an octahedral sheet. If M^{n+} is divalent (Mg^{2+}), a *trioctahedral* or *brucite-like* sheet is produced, if it is trivalent (Al^{3+}), then 2 out of every 3 octahedral sites are occupied leaving a vacant site and the generation of a *dioctahedral* or *gibbsite-like* sheet.

Clays can be classified into 1:1 and 2:1 types according to the layering of T and O sheets. 1:1 (or T-O) clay minerals consist of a single T sheet linked to a single O sheet, and 2:1 (or T-O-T) clay minerals consist of a single O sheet sandwiched between two T sheets (Figure 2).

In the case of 1:1 clay minerals, each particle consists of 1:1 layers stacked one above the other, with the half unit formula $M_xSi_2O_5(OH)_4$. For *dioctahedral* species, $M_x = Al_2$ (e.g. kaolinite and halloysite) while in case of *trioctahedral* species $M_x = Mg_3$ (e.g. serpentine) (Figure 2). The 1:1 layers are electrically neutral and weak hydrogen bonding and van de Waals forces hold the adjacent layers. Given no isomorphous substitution takes place in the

1:1 layer, these clay minerals carry no structural (permanent) charges and the total layer charge is only accounted for by edge (pH-dependent) charges. Consequently, their cation exchange capacity (CEC) is typically low (<10 meq/100 g) and these clay minerals do not undergo interlayer swelling in water making them of less interest for biomedical application than the 2:1 class. One exception in this regard is halloysite, whose hydrated 1-1 sheets roll up into nanotubes/nanocylinders giving rise to a higher specific surface area (SSA) and total pore volume[27] that confers several interesting possibilities, particularly for intracellular drug delivery[28].

For 2:1 clay minerals, each particle consists of 2:1 layers stacked one above the other, with the half unit formula $M_xSi_4O_{10}(OH)_2$. In the case of *dioctahedral* species, $M_x = Al_2$ (pyrophyllite) while in the case of *trioctahedral* species $M_x = Mg_3$ (talc). Pyrophyllite and talc are of low reactivity (SSA ≈ 20 m^2/g) and their layered structure is electrically neutral. In contrast, smectites, mica and vermiculite groups are characterized by isomorphous cation substitutions in the T and/or O sheets producing clay particles with a wide range of net surface charges, ion exchange capacities, surface reactivity, swelling and gelation properties.

The smectite group is represented by two key clay minerals: dioctahedral montmorillonite (MMT) $Na_m(Al_{2-m}Mg_m)Si_4O_{10}(OH)_2.nH_2O$ and trioctahedral Laponite $Na_h(Mg_{3-h}Li_h)Si_4O_{10}(OH)_2.nH_2O$ (a synthetic Hectorite manufactured by BYK-ALTANA), which are the most investigated among all phyllosilicates in relation to biomaterial design (Figure 2). Their relatively low net charge (0.2 – 0.6 / structural formula unit) allows smectite platelets to undergo complete dissociation (delamination) by osmotic swelling enabling a rich surface chemistry [29]. Laponite in particular is notable for: i) its small particle size of 25-30 nm diameter and 1 nm thickness[30] yielding a high specific surface area (800 m^2/g) and cation exchange capacity (80 - 150 meq/100g)[31,32] and, ii) its charge anisotropy and heterogeneity in the form of a permanent negative charge on the surface and positive (amphoteric) charges on the edges [33]. These features result in a rich array of possible interactions with biomolecules or polymers, and yield unique rheological properties following dispersal in water, including the generation of arrested gel or glassy states[34].

In contrast to the smectites, vermiculites display a higher layer charge (0.6 – 0.9 / unit structure) which restricts water accessibility in the interlayer region. As a result vermiculites undergo crystalline, rather than osmotic, swelling and do not undergo delamination[31]. Nevertheless, vermiculites possess a high SSA (750 m^2/g) and high CEC (120 – 200 meq/100g). Similarly, illites possess a still higher net charge (0.8 – 1.0 /unit formula) which further reduces swelling and thus yields a low SSA (30 m^2/g) and low CEC (10 - 40 m^2/g) [31,32].

Finally sepiolite and palygorskite (Figure 2) are distinct from other clay minerals due to their inverted 2:1 ribbons (polysomes) which feature rectangular channels running parallel to the opposing ribbons, thus giving these clay minerals a fibrous morphology with high SSA (≈900 m^2/g) and high surface reactivity[35,36]. The rheology of sepiolite and palygorskite depends on physical entanglement and so, in contrast to other clays, is relatively stable at different ionic strengths and pH[35,36].

3. Clays in biomaterial design

The surface reactivity of clays, in particular the high adsorption and exchange capacity, specific surface area and charge heterogeneity described above, allow for a range of possible

interactions of relevance for biomaterial design. This includes interactions with synthetic scaffold materials, organic and inorganic components of the extracellular matrix and soluble factors as well as direct interactions with cell surfaces and intracellular signalling pathways. Before exploring cell-clay interactions we briefly survey the relevance of clay interactions with polymers, proteins and minerals for biomaterial design.

3.1 Clay-polymer interactions for scaffold design

The potential of polymer-clay nanocomposites to achieve materials with greatly improved mechanical properties is evidenced by the significant volume of literature and their use across almost the full scope of modern material applications [37]. In the context of biomedical applications, inorganic clay mineral (along with silicon and calcium phosphate) nano-phases are increasingly being incorporated into polymers with established biocompatibility to enhance the mechanical and degradation properties of the polymeric base. For example, clay nanoparticles can act as physical cross-linkers in hydrogels that combine the dynamic properties of physical gels, such as self-healing for minimally-invasive delivery, with significantly improved mechanical strength and toughness [6,11,38,39,40] . Clay nanoparticles can also significantly improve the mechanical strength, toughness and degradation properties of hard scaffolds, allowing the generation of porous matrices with mechanical properties that approximate that of bone matrix[10,41,42,43].

The ability of clay minerals to interact with polymeric matrices has been reported for both uncharged as well as positively and negatively charged polymers through various mechanisms[24,44 ,45]. The negative silanol groups on the external surface of clay minerals are the main sites of electrostatic interaction with cationic groups on positively charged polymers, which are able undergo intercalation/exfoliation between clay layers. On the other hand, negatively charged polymers tend to be adsorbed via electrostatic interactions on the positive rims of clay minerals and/or through cation bridging on the negative clay surfaces, although ligand exchange and van der Waals interactions also may play a role.

Recent approaches have employed the dynamic mechanical properties conferred by clay nanoparticles in the optimization of 3D printing inks to allow the formation of tough hydrogels in various complex structures by controlling the pre-gel solution's viscosity and shear thinning properties as a function of clay loading. For example, recent studies have shown that methylcellulose-alginate-nanoclay hydrogels could be 3D printed into various biocompatible constructs of clinically-relevant, preserved shapes[46]. Indeed, the thixotropic and yield stress properties of the higher concentration (>5 weight percent) colloidal Laponite gels alone were themselves found to allow the printing of self-supporting structures in air without the need for a crosslinking water bath[47].

3.2 Clay-biomolecule interactions for delivery of soluble factors

The capacity of clay particles to bind biological molecules has been known by scientists for over fifty years. Clinicians observed that the presence of certain drugs in the blood stream was severely reduced when patients simultaneously received clay-based anti-diarrheal treatments[48]. This was found to result as a consequence of binding of drugs by the clay particles. This property is now utilised in the design of tablets to carefully control the release and action of a range of drugs.

Various mechanisms underlie clay associations with biomolecules. These can include intercalation within the interlayer gallery through cation exchange reactions, adsorption via

electrostatic interactions on their positive and/or negative surfaces, binding of polar biomolecules at hydrophilic (octahedral) and hydrophobic (tetrahedral) sites, as well as ligand exchange, cation/water bridging, hydrogen bonding and van der Waals interactions.[1,49] Such utility has been extensively explored in the development of drug delivery systems as for example in the use of smectites for controlled release of ibuprofen[50], Donepezil[51], nicotine[52], timolol[53] and many others[1,23,54]. From a regenerative medicine perspective, we have shown the potential for clay nanoparticles to self-organise, via electrostatic interactions, into gels under physiological conditions. These clay hydrogels display the ability to take-up and bind bioactive molecules to direct the differentiation of endogenous cell behaviour or cell populations encapsulated within the gel (Figure 3). This approach has been applied to initiate the formation of new blood-vessels at an injury site through localisation of vascular endothelial growth factor[15] and to induce bone at significantly reduced doses of an osteoinductive (bone morphogenetic protein) growth factor[16].

The data for the use of pure Laponite gels as growth factor delivery vehicles suggests rather minimal release of clay bound molecules making their bioactivity dependent on the invasion of responsive cell populations into the gel itself. While this ability to sustain a localized regenerative microenvironment may have advantages in certain contexts, other clinical scenarios require sustained release of a molecule to the surrounding tissue. Such an effect can be achieved by combining clays with polymers to form a nanocomposite for the purpose of modified drug delivery[54,55,56]. For example, a recent study observed negligible release of fibroblast growth factor-2 (FGF2) from pure Laponite gels, but achieved a tuneable release profile with varying concentrations of the glycosaminoglycan, heparin which associated with Laponite to form a shear-thinning (and thus injectable) hydrogel. Heparin itself has a physiological role in binding biological molecules which resulted in a bi-modal effect of heparin concentration on FGF2 release implying competitive binding between the clay, the polymer and the growth factor [57]. As well as smectites, halloysite nanotubes (HNTs) have also been used for modified drug release, for example to achieve sustained release of dexamethasone[58]. Such studies underline the potential of clay-based strategies for modifying the release of bioactive agents to initiate and sustain regenerative responses at sites of injury or disease.

3.3 *Clays and biomineralisation*

A common feature of enhanced osteogenesis in association with clays (reviewed below) is a strong and early enhancement in calcium phosphate (CaP) mineralisation, suggesting that clays may have a direct effect on CaP nucleation, growth and/or deposition[,4,5,6,9,12,59,60]. For example, the culture of skeletal populations on Laponite-containing nanocomposites yielded almost double the mineralised matrix in comparison to controls[4,59]. A few studies have also provided evidence for the intrinsic 'bioactivity' of certain clays, understood in this context as the specific ability to initiate biomineralisation in simulated body fluid (SBF). Laponite addition to (poly)caprolactone (PCL) electrospun fibres increased CaP deposition[61] and a sintered Laponite bioceramic induced hydroxyapatite formation and deposition on its surface after 7 days in SBF[62].

Ambre and Katti et al.[63,] have sought to enhance the utility of clay for mineralisation through the development of MMT clays modified with an amino acid (5-aminovaleric acid). Initially applied as a biocompatible organic modifier for the development of a polymer-clay nanocomposite[64], functional groups on the amino acid modifier were able to support the nucleation and precipitation of hydroxyapatite to generate a biomineralised MMT

hydroxyapatite hybrid material. This material has subsequently shown promise for bone tissue engineering applications [12,65,66]. The mechanism for clay mediated biomineralisation remains poorly understood. The anisotropic and heterogeneous charge structures of clay particles and their aggregates, as well as the affinity of silica for calcium ions[67,68] may provide favourable nucleation sites facilitating adsorption of Ca^{2+} and HPO_4^{2-} to reduce the energy barrier for calcium phosphate deposition [69].

4. Clay – Cell Interactions

The well-established ability of clays to interact with drugs and other biological molecules for controlled delivery and their ability to interact with biomedical polymers for enhanced mechanical properties have driven a growing interest in the utility of clays in biomaterial design. As a result, a growing number of studies have investigated the biocompatibility of clays and their direct interactions with cells and tissues. These studies have yielded unanticipated observations of clay dependent enhancements to cellular responses such as cell adhesion and differentiation. Before turning to examine the evidence for such enhancements it is important to review the evidence for the biocompatibility of clay nanomaterials and their uptake by cells.

4.1. Cellular uptake of clays

Following release from a degrading polymer matrix or via addition as a dispersion to cell culture media, cells are likely to encounter clays as free-floating particles or aggregates. Clay particles are characterized by their nanoscale size and anisotropic charge distribution and thus consideration of cellular-nanoparticle responses are relevant. Since cellular physiological responses are directly and strongly affected by their uptake of nanoparticles[70,71], understanding the extent and mechanism by which clay nanoparticles enter cells is of importance to understanding their bioactivity and may present further opportunities for tissue regeneration applications.

Nanoparticle cell interactions are highly dependent on particle size, shape and charge[72]. The optimal particle size for cellular endocytosis is in the order of 25 – 30 nm[73] which suggests Laponite particles in particular (25 – 30 nm diameter sheets) are likely to be endocytosed, and several lines of evidence support this. Confocal analysis following addition of dispersed Laponite to adipose derived stromal cells indicated internalisation and cytoplasmic distribution of clay particle/aggregates but with some accumulation on cell membranes[5]. The addition of colchicine (to impede clathrin-mediated endocytosis) resulted in a reduction of the number of cells associated with rhodamine-labelled Laponite as measured by flow cytometry. From this reduction of Laponite associated cells, the authors inferred an internalisation efficiency of at least 40% (depending on Laponite concentration) and confirmation of a role for clathrin-mediated endocytosis in cellular uptake[5] (Figure 4).

Larger sized MMT particles (100s nm diameter) were shown to interact with cell membranes of Chinese Hamster Ovary cells at a concentration similar to that reported for Laponite (100 μg/ml) but with no clear evidence of cellular uptake [74]. Halloysite nanotubes, on the other hand, vary in length between 1-15 microns but, presumably due to their high aspect ratio were efficiently internalised into the cytoplasm of both MCF-7 and HeLa cell lines without modulation of cell phenotype or preventing cell proliferation up to a concentration of 75 μg/ml [75].

As well as particle size and shape, direct interaction of the cationic edge charges of the clay nanoplatelet with the anionic glycoproteins and phospholipids of the cell membrane may also facilitate cellular transport and uptake,[5,70,76,77]. Layered double hydroxides are characterised by the general formula $[M^{II}_{1-x} M^{III}_{x} (OH)_2]^{x+}[A^{m-}_{x/m}.nH_2O]^{x-}$, in which the isomorphous substitution of M^{II} by M^{III} gives the brucite-like layers positive charges rather than a negative surface charge as in the case of cationic clays[78]. These positively charged particles were found to form electrostatic interactions with anionic cell surfaces to facilitate receptor-mediated endocytosis[79,80].

Other studies have shown that internalization of clays is not restricted to receptor mediated endocytosis. Smirnov et al found that the addition of chloroquine (an endocytosis inhibitor) resulted in a 20% reduction of sepiolite internalization efficiency by mammalian cells and amiloride (a micropinocytosis inhibitor) reduced the sepiolite internalization efficiency by 50% suggesting micropinocytosis to represent the main cellular uptake pathway of sepiolite nanofibers[81]. Time-lapse video microscopy showed spontaneous internalization/exclusion of sepiolite by the cells and, interestingly, its transmission between neighbouring cells[81].

Understanding the fate of clay nanoparticles following cellular uptake including their degradation profiles and the extent of endosomal release into the cytoplasm will be important for elucidating mechanisms behind clay bioactivity and resolving ongoing questions regarding cytocompatibility.

4.2 Toxicology of clays

Most toxicology studies to date have demonstrated negligible effects of clay nanoparticles on human or animal cells at relevant physiological concentrations. Oral ingestion of MMT did not affect the mortality of exposed Sprague-Dawley rats up to a dose level of 5700 mg/kg body weight (LD50 >5700 mg/kg)[74]. In another study, MMT orally administered at doses up to the highest level tested (1000 mg/kg) indicated rapid clearance within 2 hours and no clay accumulation observed in the long term in any specific organ[82]. An increasing number of studies reporting implantation of smectite based biomaterials have yet to note any indication of toxicity or persistent inflammation[7,9,10,83,84] . For example, unlike carbon nanotube based composites MMT-based composites did not increase local vascularization and pro inflammatory markers against controls following subcutaneous implantation[85].

When applied directly to cells *in vitro*, Laponite NPs dispersed in cell culture media showed no significant effect on cell morphology, viability or proliferation of human bone marrow and adipose derived stromal cells up to a concentration of 100 μg/ml over 7 days [4,5]. At higher clay doses, a decrease in metabolic activity was observed. Interestingly, it is notable that the half maximal inhibitory concentrations (IC50s) determined (4 mg/ml and 1 mg/ml for bone marrow and adipose derived cells, respectively) remained around ten-fold higher in comparison with other commonly investigated nanoparticles such as hydroxyapatite (IC50 = 250 μg/ml) and silica (IC50 = 400 – 500 μg/ml) [4,5,86–88].

Likewise, *in vitro* cytotoxicity of MMT on Hamster Ovary cells (CHO) was only evident at >1 mg/ml[74]. Examination of cell viability and proliferation profiles of human dermal fibroblasts (NHDF) remained close to 100% for all MMT concentration tested (5 - 300 μg/ml)[89]. Furthermore, no or negligible genotoxic effects of exfoliated MMT were observed up to a concentration of 1mg/ml across three separate assays: i) the Comet assay (DNA

damage evaluation) on CHO cells *in vitro*, ii) the micronucleus assay (chromosomal damage evaluation) on Peripheral-blood cells *in vivo*, and, iii) the *Salmonella* gene mutation assay[74].

Some studies have however observed a more acute response following the addition of clay. A study of proliferation and colony formation of human normal intestinal cells revealed inhibition even at low concentrations of MMT (5 μg/ml) within 24 hours[82]. Another study observed a significant loss of viability in the human hepatic cell line HepG2 in response to low concentrations of both unmodified and organically modified MMT nanoclays[90]. An important factor in both these studies is the well characterised flocculation behaviour of clay colloids at increasing concentrations and in the high salt concentrations of cell culture media. In such conditions clay particles will typically aggregate into micro-sized clusters / agglomerates with a tendency to accumulate around cells. Such an accumulation can block membrane channels and impair cellular metabolism and cytoskeleton organization[4,5,91,92]. Thus, MMT was found to inhibit cell proliferation and colony formation independently of any direct effect on cell viability in the short term. This was evidenced by lactate dehydrogenase (LDH) based assessments of membrane integrity which was compromised at very high concentrations (1 mg/ml) and reactive oxygen species (ROS) generation which was observed only after 48 hours despite clear inhibition of proliferation within 24 hours[82]. The authors inferred an indirect effect of clay aggregation and accumulation on cell proliferation rather than a direct cytotoxic effect. Consistent with this conclusion is the observation that clay nanoparticle incorporation as a dispersed phase within a polymeric network allows considerably higher dispersion stability [23,24] and typically preserves good cytocompatibilty at considerably higher concentrations of nanoclay [6,9,59,60 ,93, 94].

As well as dispersion stability, particle shape and size are of importance to cytocompatibility. For example, a study observing slightly lower cytotoxicity for MMT compared with Laponite suggested the lower aspect ratio of MMT (300:1 vs 25:1) to be an important factor [95]. More pronounced is the pro-inflammatory response and lower threshold cytotoxic concentration observed for the 1:1 tubular clay mineral, halloysite. Both human epithelial adenocarcinoma (HeLa) and human breast cancer (MCF-7) cell lines maintained their viability at >70% when incubated in HNTs-containing media, at concentrations up to 75 μg/ml above which pronounced cytotoxicity was observed[75]. Another study observed good viability and membrane integrity up to Halloysite concentrations of 100 μg/ml[96,97] , but detected significant pro-inflammatory effects at HNTs concentrations as low as 1 μg/ml and significant changes in protein expression at high Halloysite content (100 μg/ml) [96]. Interestingly, surface coating of Halloysite by PEG polymer significantly improved Halloysite cytocompatibility even at much higher doses (<500 μg/ml)[98].

While the current available *in vitro* and *in vivo* studies indicate a good toleration of clay nanoparticles, even at relatively high doses if dispersion stability is maintained, long-term implantation and bio-distribution studies are warranted and remain to be undertaken. To date, there remains a paucity of information detailing the biodegradation and clearance profiles of clay nanoparticles and nanocomposites surgically implanted or parenterally delivered or indeed, their modulation of acute and chronic inflammatory events. In addition, exposition of the mechanisms behind the cytotoxic effects observed *in vitro* and the relative importance of dispersion stability, surface charge, ion exchange capacity and particle size and morphology on such dose-dependent effects remains to be demonstrated.

Despite some ambiguity regarding the cytotoxic assessment of clay nanoparticles, a growing number of studies exploring the biocompatibility of various polymer-clay nanocomposites

have observed, not only minimal cytotoxicity but direct clay-dependent enhancements to cell functions such as cell adhesion, proliferation and differentiation offering new potential biomedical applications.

4.3 Cell adhesion and proliferation

Poly-ethylene glycol (PEG/PEO), like most polymeric hydrogels, is hydrophilic, non-fouling and does not support protein and cell adhesion[99,100]. Laponite incorporation in PEG hydrogels at 40-70% (wt.%) was shown to enhance cell adhesion, spreading and proliferation of NIH 3T3 mouse fibroblasts[93,101], MC3T3-E1 mouse preosteoblasts[59,102] , and human bone marrow stromal cells (hBMSCs) [103] in a clay dose-dependent manner. Cells cultured on PEG/Laponite films with <40% clay showed poor cell adhesion and growth and exhibited non-adherent spherical morphology with disrupted and disorganised F-actin fibres. Following an increase in the clay fraction, however, (40% - 70%), hBMSCs were observed to readily grow and proliferate, displaying a flat morphology and resulting in a confluent monolayer after 14 days' culture [59,93,101,103] (Figure 5). 3D encapsulation of mouse embryonic fibroblasts led to vinculin expression in the PEG clay nanocomposite hydrogel but not its clay-free counterpart, indicating the formation of focal adhesions[102]. Similarly, only Laponite-containing poly-*N*-isopropylacrylamide (PNIPA) hydrogels were able to support adhesion, spreading and proliferation of HepG2 human hepatoma, human dermal fibroblasts, and human umbilical vein endothelial cells (HUVECs). In this case the effect was dose dependent up to a maximum concentration (C_{clay} of 6 x 10^{-2} molar) above which cell adhesion was impeded[104].

Similar clay-dependent effects on cell spreading and proliferation have been observed following the addition of montmorillonite to gelatin-cellulose [105], polyurethane (PU)[106], and chitosan-based[107] scaffolds. In the case of chitosan, a direct comparison between an MMT based composite and a hydroxyapatite based composite revealed increased cell spreading and proliferation on the clay-based nanocomposite[108]. Halloysite nanotubes have also been employed for enhanced cell attachment in nanocomposites to similar effect. Enhanced cell spreading was observed after surface treatment of a polyelectrolyte film with halloysite compared with MMT. Interestingly, with both treatments improved adhesion and proliferation was observed against untreated controls[109]. Dose-dependent positive effects on adhesion and proliferation were also observed upon addition of halloysite to poly(vinyl alcohol)[110] and alginate[111] based nanocomposites.

Various factors have been suggested as possible mechanisms behind clay-enhanced cell adhesion and spreading. Indirect enhancement of cell adhesion via the adsorption of cell adhesive proteins such as fibronectin or vitronectin from serum supplemented media is frequently cited and likely to play a role [103,104,112,113]. Interestingly though, even in serum-free media, fibroblast attachment and spreading was observed on PEO/clay surfaces following a clay concentration dependent trend. This contrasts with PEO alone or, indeed, standard tissue culture plastic, which typically does not support cell attachment or spreading in the absence of serum [93] suggesting that direct clay-cell interactions facilitate cell adhesion. Clay nanoparticles may therefore act directly as focal adhesion sites through the provision of reactive functional groups (e.g. >Si-OH_2^+) for cell attachment [103, 104, 113]. Alternatively, in some cases, the particular hydrophobicity /hydrophilicity balance between hydrophobic polymer chains and the hydrophilic clay dispersion could directly mediate cell adhesion as well as promote protein adsorption[104, 114,115]. Another possible mechanism could be increased local concentrations of divalent cations such as Ca^{2+} or Mg^{2+} which exchange on clay

surfaces preferentially over monovalent ions given their increased charge density. Such divalent ions are essential for the function of integrins, the transmembrane receptors that mediate cell interactions with ECM[116]. It has also been suggested that Mg^{2+} ions arising from the dissolution of Laponite could promote cell adhesion [112,117], however, the concentration of clay dissolution expected in physiological buffers is disputed[118] and the concentration of divalent ions in cell culture media is also unlikely to be limiting.

Finally, modification of clays with exchangeable organic compounds, an approach widely used to improve the intercalation of non-polar polymers for nanocomposites, has also been explored as a means to biochemically functionalise clays to modulate adhesion and other cell functions. Bongartz and Barlas et al., reported an approach to control the capacity and selectivity of Cloisite for cell adhesion and proliferation via folic acid modification[119,120]. In contrast to A549 cells (FA receptor-negative), the adhesion and proliferation behaviour of HeLa cell line (FA receptor-positive) showed a FA-dependent enhancement compared to the unmodified Cloisite[119]. Similarly, intercalating polydopamine into clay nanosheets significantly improved the cell affinity of clay-polyacrylamide hydrogels compared to polydopamine unmodified controls[121]. Such approaches suggest new avenues for modulating clay based nanocomposites to achieve specific cell responses[121].

4.4 Clays and osteogenesis

In addition to the utility of clays for drug and growth factor delivery, various studies have highlighted a direct bioactive effect of clays on the osteogenic differentiation of skeletal populations. Gaharwar and colleagues reported an improved osteogenic response of preosteoblast cell line MC3T3[6,10,59,122,123] and bone marrow stromal cells[9,61,103] as a direct function of modulating Laponite content in PEO[59,103], PGS[10,122] , PEG[123], PCL[61] and GelMA[6,9] polymer nanocomposites. The authors found increasing Laponite concentrations from 40% to 70% in PEO led to a 10-fold increase in alkaline phosphatase (ALP) activity and CaP mineralisation by day 28 as well as a significant rise in osteogenic-related gene expression[59]. Likewise, GelMA-clay nanocomposite hydrogels were able to support osteogenic differentiation of both surface seeded[6] and encapsulated[9] skeletal populations. Su et al observed enhanced ALP activity and bone-related gene expression in silk fibroin hydrogels with addition of Laponite[11]. The bioactivity of clay in its dispersed form has also been convincingly demonstrated. Dispersion of Laponite in cell culture media up to a concentration of 100 μg/ml resulted in a dose-dependent and stage specific upregulation of osteogenic gene expression (*RUNX2*, *BGLAP* and *SPARC*), increased ALP activity, type I collagen synthesis and CaP deposition [4,5]. Interestingly, the osteogenic effects were apparent even in the absence of the standard osteogenic differentiation media supplements dexamethasone, ascorbate-2-phosphate and beta-glycerophosphate suggesting the possibility of direct interaction with select osteogenic pathways.

Several groups have also demonstrated a similar osteogenic effect for other clay mineral types in nanocomposite biomaterials. For instance hBMSCs cultured on silk/MMT surfaces showed an up-regulation in osteogenic gene expression in a clay dose-dependent manner[60]. Ishikawa and colleagues found imogolite addition resulted in increased ALP activity and mineralization by MC3T3s [124], compared to carbon nanotubes which did not affect differentiation over TCP controls. The incorporation of HNTs within PCL scaffolds resulted in a doubling of hBMSC ALP activity[125] and 1% HNTs increased hBMSC osteogenic gene expression over pure PCL control, equivalent to that seen with the addition of 5% nano-hydroxyapatite [126].

The mechanism(s) behind clay promotion of osteogenic differentiation remain poorly understood. The known osteogenic effects of clay degradation products are frequently cited [4,5,6,14] In the case of Laponite, $Si(OH)_4$, Mg^{2+}, and Li^+ have each been associated with enhanced osteogenic cell function. For example, magnesium ions are involved in activating osteogenesis-regulating pathways ((HIF-1α and PGC-1α)[127,128] and are essential for integrin adhesion to biomaterial surfaces [129], orthosilicic acid promotes collagen type 1 synthesis and osteoblast differentiation[130] and lithium is known to activate canonical Wnt-responsive osteogenic genes through the inhibition of GSK3β[131,132]. However, the rate and extent of clay dissolution within endosomal (or lysosomal) intracellular compartments, or in cell culture solutions, remains to be confirmed. In addition osteogenic effects have been seen using clays such as MMT[12,60], Halloysite[125,133] and attapulgite[134] each with different dissolution products. Wang et al. observed similar osteogenic effects with addition to electrospun PLGA nanofibers of both Laponite[135] and the aluminium phyllosilicate, attapulgite[134] also in the absence of additional osteogenic culture additives. In both these studies, clay addition resulted in improved surface hydrophilicity and mechanical properties of the PLGA nanofibers which had a clear effect on cell adhesion and proliferation compared to pure PLGA – factors that are also likely to contribute to a stronger osteogenic response.

Various other models for how clays may influence differentiation pathways independent of clay dissolution could be proposed (Figure 6)**.** Modulation of local calcium phosphate dissolution/formation dynamics is known to play an important role in the osteogenic activity of mineralised (or mineralising) biomaterials such as ceramics and bioactive glasses suggesting one potential mode of osteogenic action for bioactive clays[136]. Furthermore, intracellular accumulation of calcium phosphates is known to play a role in both mineral deposition[137] and osteogenic differentiation[136], and thus intracellular delivery of calcium phosphate nanoparticles was able to promote osteogenic commitment in skeletal populations[138]. It is therefore possible that cellular uptake of clays may aid the transport of calcium phosphate minerals and/or their ions to promote these pathways.

Similarly, clay-protein interactions, in addition to their ability to stabilize extracellular growth factor concentrations[15,16] may also enhance osteogenesis by aiding the cellular uptake of bioactive molecules. Such utility has been applied directly through the use of clays as nanocarriers for drugs and plasmids[23,139]. Intracellular clay-protein interactions may also directly influence intracellular signalling events following clay uptake, for example through catalysis via co-localisation of an enzyme and its substrate. To provide one rather striking example, a study seeking to mimic pre-cellular biochemical processes during early-life evolution found that Laponite clay gels were able to consistently enhance the transcription and translation of nucleic acids (a process involving more than 30 enzymatic reactions) in a cell lysate solution compared to clay free controls[140].

Despite considerable interest in the osteogenic activity of certain nanoclays, the effect of their chemical composition and physicochemical properties on osteogenesis remains unexplored. Investigating the specific contributions of clay structural/compositional parameters (e.g. particle size, surface charge and surface area) on clay rheology and bioactivity may provide further insights into underlying mechanisms and allow optimisation for greater clinical usefulness.

5. Conclusion & Future Directions:

The current review has sought to highlight the opportunities presented by clay nanoparticles, composites and hydrogels for biomaterial design and discussed the potential mechanisms for clay bioactivity. The high surface reactivity of clays and their wide range of possible interactions with polymers, proteins and minerals makes this an exciting and fertile field of research for biomaterial design which, to date, remains relatively unexplored. An increasing body of evidence for clay bioactivity through influence over the organic and inorganic extracellular environment and through direct interaction with intracellular processes raises new questions and presents new opportunities for regenerative medicine.

A priority for future studies will be to dissect the various modes of influence clay interactions exert on cellular activities and cell differentiation profiles. In this review we have discussed the potential of clays to interact directly with cell receptors to mediate cell adhesion as well as to influence intracellular pathways via their uptake into cells. We have also noted the affinity of clays for polymers and their ability to modify the surface and mechanical properties of biomaterials – both key for cellular differentiation and function. We have described studies demonstrating the influence of clays on mineralisation processes which themselves exert upstream influences on cellular differentiation. Finally, the affinity of clays for proteins and their potential for stabilizing extracellular biochemical cues to induce specific regenerative responses has been highlighted.

As well as resolving the relative importance of these mechanisms, it will also be important to understand how key clay physicochemical properties may influence these pathways and biomaterial parameters more broadly. Subtle changes in compositional and structural parameters of clays and their colloids will profoundly influence the various interactions we have described. For example, we noted that the colloidal properties of clay minerals strongly depend on clay concentration, salinity of the medium, particle size and isotropy, layer charge and the available interlayer cation[29]. The possible role of clay amphoteric edge charges on cellular uptake and cell adhesion will be critically dependant on ambient ionic and pH conditions [141,142] and interactions with proteins, polymers and other minerals may be tuned through modulation of clay structure and cation exchange capacity[49]. Greater understanding of these structural/compositional influences will allow greater control over the stem cell microenvironment/niche and will be key to successfully harnessing the unique opportunities afforded by clay chemistry for biomaterial design, regenerative medicine and, ultimately, patient benefit.

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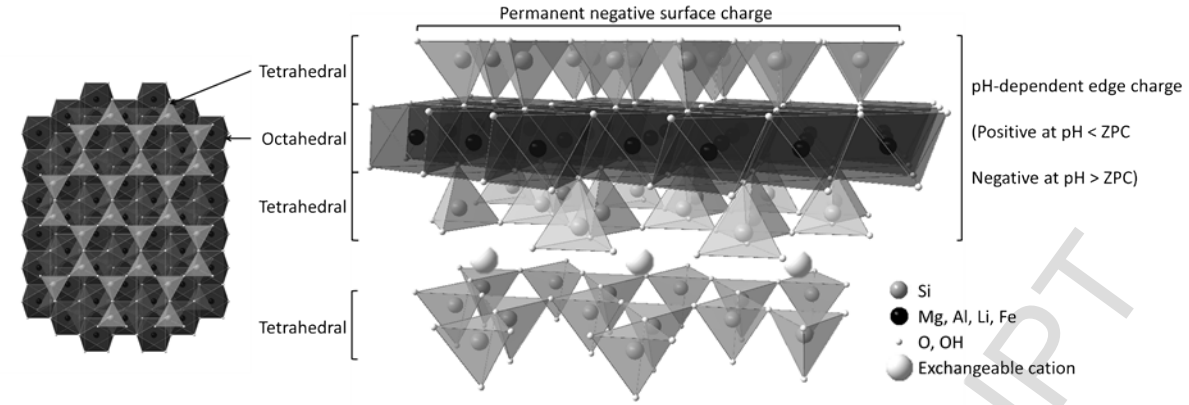

Figure 1. The structure of smectites. Clays are formed of layered tetrahedral (T) and octahedral (O) sheets. In the case of smectites an octahedral sheet of metal oxides (usually Mg^{2+} or Al^{3+}) is sandwiched between two tetrahedral silica sheets. Two types of charges originate on smectite clay particle: i) permanent negative charges on the surface due to isomorphous cation substitution in the tetrahedral and/or octahedral sheets (e.g. Li^{+} for Mg^{2+} in Laponite) balanced by exchangeable cations such as Na^{+} or Ca^{2+} in the interlayer gallery. ii) positive (amphoteric) charges on the edges due to broken Si-O, Al-OH and Mg-OH groups. At pH < Zero Point of Charge (ZPC), these edge charges become positive with anion exchange capacity while at pH> ZPC they become negative with a cation exchange capacity. Adapted with permission[1]. Copyright 2014, John Wiley and Sons.

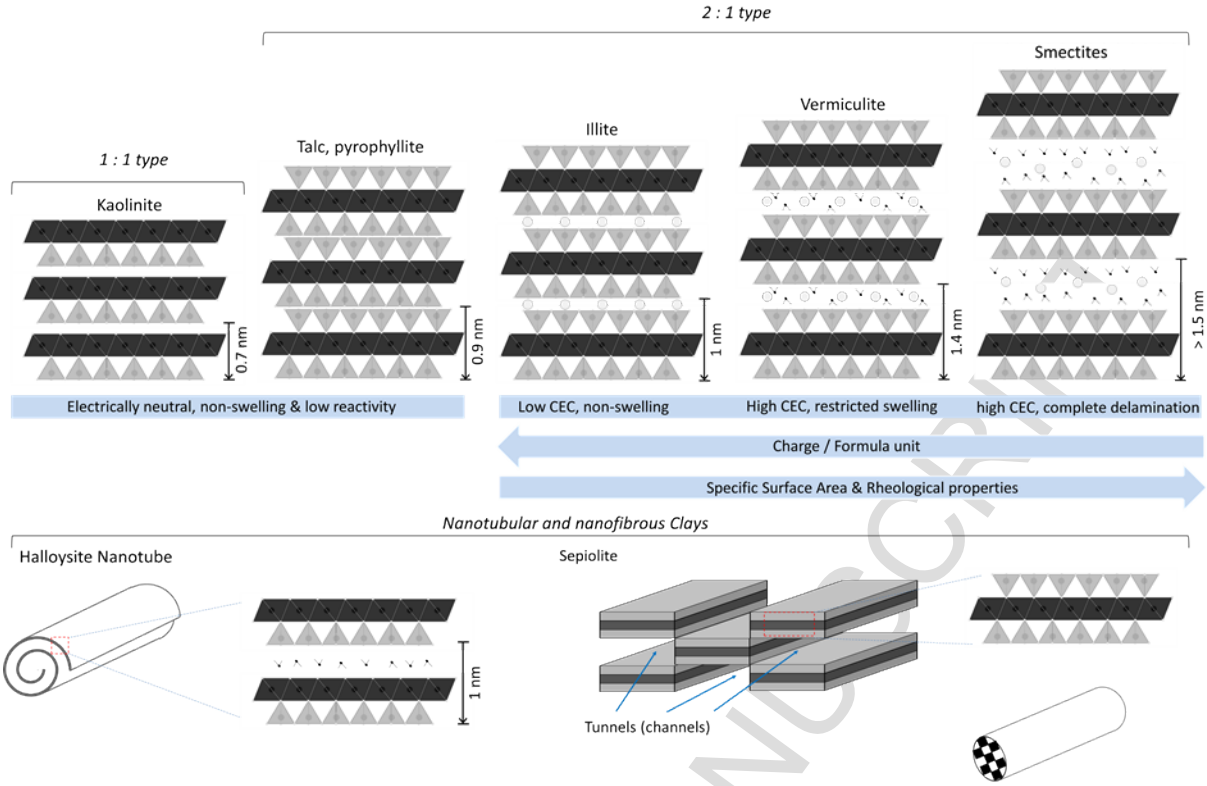

Figure 2. Clay structure and reactivity. The reactivity of clays is largely a function of their swelling capacity. Kaolinite (of the 1:1 clay family) and talc and pyrophyllite (of the 2:1 clay family) possess no structural charges and consequently are non-swelling and of low adsorption capacity. The high layer charge on vermiculite and illite restrict their swelling and gelling tendency although their surface area and CEC are relatively high. Smectites are characterized by their relatively low layer charge which allow their particles to undergo complete dissociation in water and give them interesting rheological/gel forming properties and surface reactivity. Halloysite is formed of hydrated 1:1 layers which role up into nanotubes (alumina sheet on the inside and silica sheet on the outside surface) and sepiolites (and palygorskite) are characterized by their inverted 2:1 ribbon structures. Such arrangements confer large SSA, porosity and sorptive capacity. Adapted with permission[1].

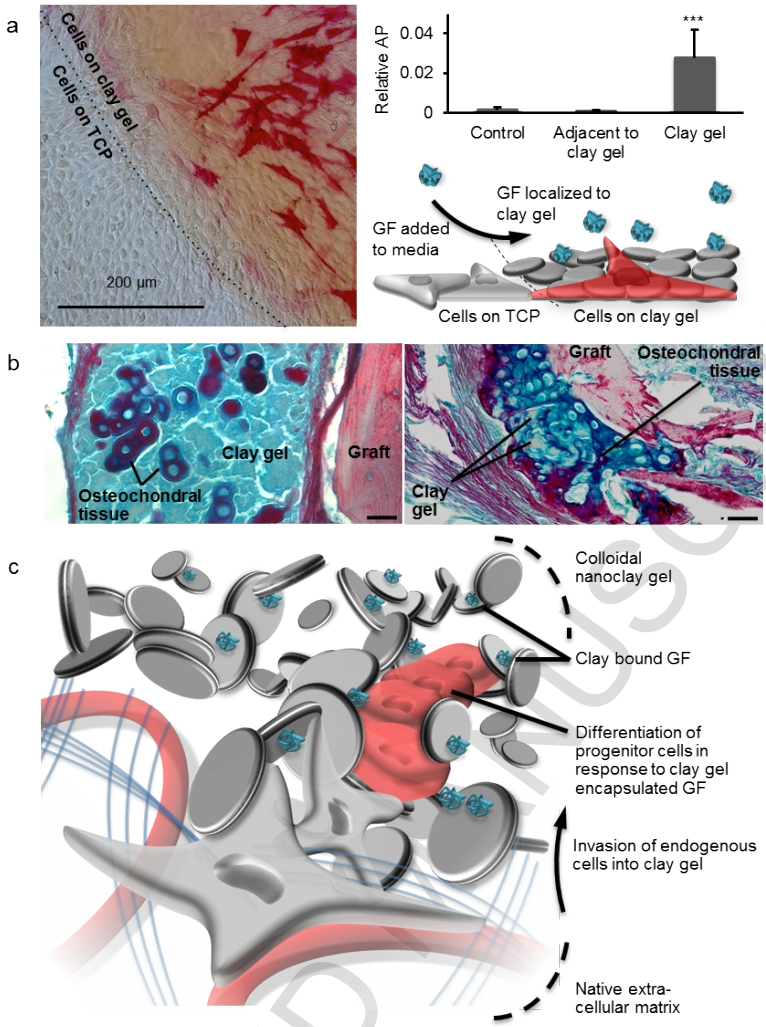

Figure 3. Clay gels for growth factor localisation. Laponite gels display the ability to take-up and bind bioactive molecules to direct the differentiation of endogenous cell behaviour. Clay gel films localize otherwise sub-efficacious doses of BMP2 to induce a) osteogenic differentiation of C2C12 myoblasts *in vitro* and b) clay-gel localised endochondral ossification *in vivo*. Scale bar = 50 µm. c) Schematic representation of proposed mechanism for endogenous stem/progenitor cell differentiation in response to clay-mediated growth factor delivery and localization (cells and clay particles not to scale). In contrast to conventional drug release strategies, the growth factor remains localised within the clay gel requiring invasion of endogenous cells from native tissue. This allows a highly localised response to the growth factor and for templating by the clay gel of new tissue formation. Adapted with permission[16]. Copyright 2016, Elsevier.

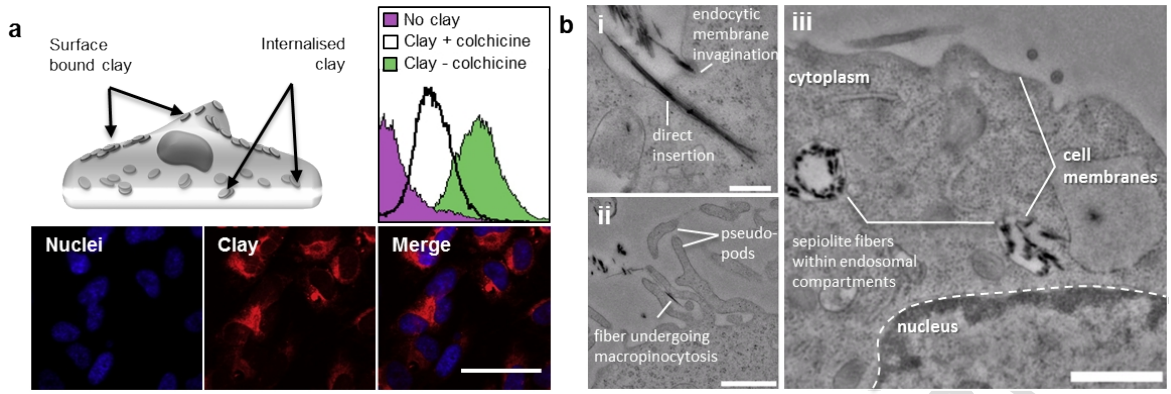

Figure 4. Cellular uptake of clay. a) Laponite directly interacts with stem cells through both attachment to the cell surface and wide distribution in the cell cytoplasm. Rhodamine-labeled laponite nanoparticles (10 μg/ml) locate around the nucleus of hASCs following 24 culture. Scale 50 μm. A significant reduction in clay cellular uptake is observed as a result of an endosomal inhibitor. b) TEM of sepiolite uptake shows endocytic and direct routes of uptake (i) as well as evidence of macropinocytosis via psuedopod formation (ii). Cytoplasmic regions clearly show sepiolite fibers within endosomal compartments. (a) Adapted with permission[5]. Copyright 2014, Elsevier. (b) Adapted with permission under Creative Commons CC-BY license by F. Castro-Smirnov et al. 2017[81].

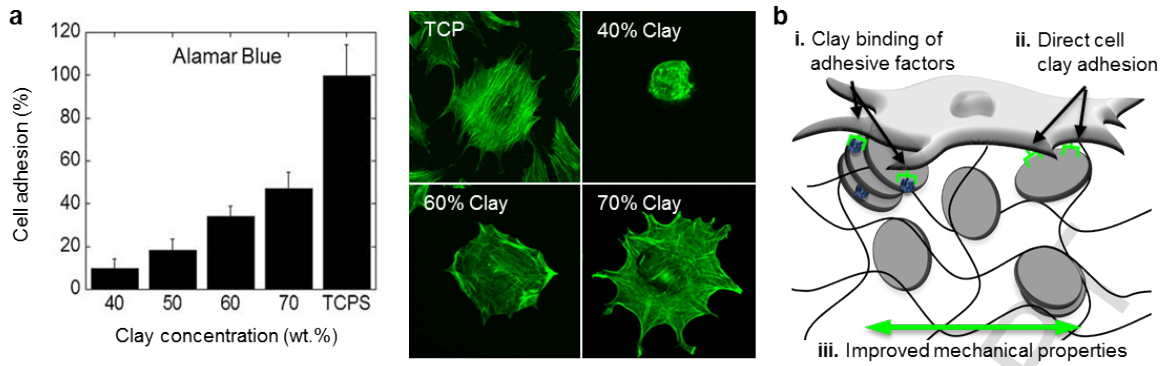

Figure 5. Clay mediated improvements to cell adhesion and spreading. a) The incorporation of clay nanoparticles in PEG polymeric hydrogel, which alone is non cell-adhesive, significantly improves hBMSCs adhesion and spreading in a dose-dependent manner. b) Schematic showing possible mechanisms of clay-enhanced cell adhesion and spreading: i) clay nanoparticles adsorb cell adhesive proteins from serum (indirect effect), ii) clay nanoparticles themselves act as focal adhesion sites thus facilitating cell attachment and spreading through a direct clay-cell interaction, iii) clay nanoparticles confer improved stiffness or other physical properties that promote cell spreading. Adapted with permission[59,103]. Copyright 2011, Elsevier and 2012, John Wiley and Sons.

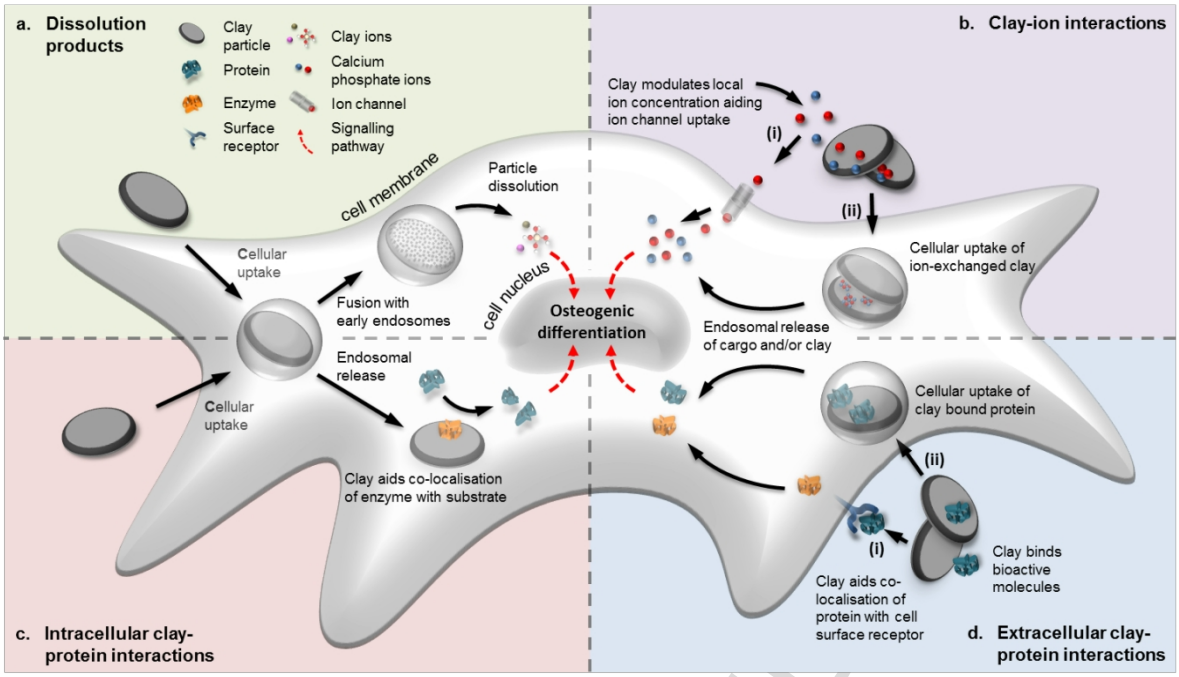

Figure 6. Possible modes of action for clay bioactivity. Clay enhancement of osteogenic differentiation of responsive populations could be mediated via various possible routes. **a.** Following cellular uptake clay nanoparticles may undergo degradation within the low pH endosomal or lysosomal intracellular compartments to release dissolution products ($Si(OH)_4$, Mg^{2+}, and Li^{+}) known to influence osteogenic cell function; **b.** Clay nanoparticles may facilitate the transport of extracellular Ca^{2+} and PO_4^{3-} ions across the cell membrane by modulating extracellular ion concentrations (i) or via uptake of ion-exchanged particles (ii) to promote mineralisation. **c.** Internalised clay nanoparticles may modulate intracellular signalling pathways through clay-protein interaction, as for example through alteration of enzyme activity as a result of adsorption/immobilization on clay surfaces. **d.** Clay nanoparticles may aid receptor interaction (i) or uptake (ii) of bioactive molecules.

Table 1: Key clay mineral species explored for tissue engineering and regenerative medicine applications with their relevant structural/compositional properties

Family	Group	Species	Chemical formula	Charge/formula unit	CEC	Particle size (nm)
1:1	Serpentine-kaolin	Halloysite	$Al_2Si_2O_5(OH)_4.nH_2O$	~0[25]	~10 meq/100g[31,32]	Nanotube diameter of ~50 nm, lumen of ~15 nm and length of ~1μm[28,58]
2:1	Smectites	Montmorillonite	$Na_m(Al_{2-m}Mg_m)Si_4O_{10}(OH)_2.nH_2O$	~0.2-0.6[25]	~80-150 meq/100g[31,32]	~80-300 nm diameter & ~ 1 nm thickness[74,95]
		Laponite (synthetic hectorite)	$Na_h(Mg_{3-h}Li_h)Si_4O_{10}(OH)_2.nH_2O$			~25-30 nm diameter & ~ 1 nm thickness[30,34]
	Sepiolite-palygorskite	Sepiolite	$X^*(Mg, Al, Fe^{3+})_4(Si, Al)_6O_{15}(OH)_2.nH_2O$	-	~4-40 meq/100g[35,36]	Nanofiber diameter of ~15 nm and length of ~200-400 nm[35,81]

CEC = cation exchange capacity; X* = traces of compensating cations (K^+, NH_4^+, Ca^{2+}, …)

Table 2: Key clay mineral species used for tissue engineering and regenerative medicine applications with their mode of presentations, and cellular interactions / effects.

Species	Mode of presentation	Studies demonstrating cellular interactions / effects			
		Cyto-compatibility	Cellular uptake	Enhanced adhesion/ proliferation	Enhanced differentiation
Halloysite	Dispersed particles, PCNs	75,83,96,97,98	75	109,110,111	125,126,133
Montmorillonite	Dispersed particles, PCNs	60,74,82,85,89,90,91,92,94,95	74	105,106,107,108,109,119,120	12,60,63,65,66
Laponite	Dispersed particles, colloidal gels, PCNs	4,5,6,7,9,10,59,84,93,95	4,5	5,59,93,101,102,103,104,112,113,117,121	4,5,6,9,10,11,15,16,59,61,62,103,122,123,135
Sepiolite	Dispersed particles	81	81		

PCNs = Polymer-clay nanocomposites

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