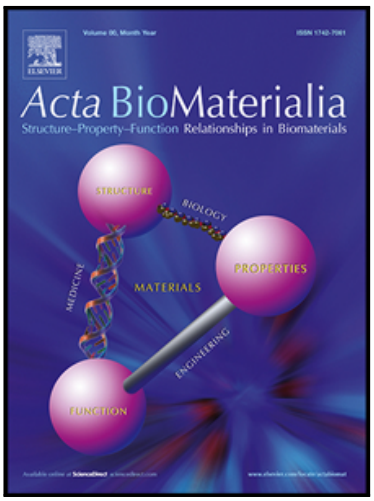


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# Injectable nanoclay gels for angiogenesis

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**Abstract**

The retention and sustained activity of therapeutic proteins at delivery sites are goals of regenerative medicine. Vascular endothelial growth factor (VEGF) has significant potential in promoting the growth and regeneration of blood vessels but is intrinsically labile. This is exacerbated by the inflammatory microenvironments at sites requiring regeneration. For VEGF to be efficacious, it may require a carrier that stabilises it, protects it from degradation and retains it at the site of interest. In this study, we tested the hypothesis that injectable nanoclay gels comprising Laponite$^{TM}$ XLG (a synthetic hectorite clay) can stabilise VEGF and retain it in the active form for therapeutic delivery. To achieve this, VEGF was incorporated in Laponite gels and its activity tested at a range of concentrations using *in vitro* cell culture tubulogenesis assays and *in*

*vivo* angiogenesis assays. We found that VEGF-Laponite gels enhanced tubulogenesis in a dose-dependent manner *in vitro*. When administered subcutaneously *in vivo*, Laponite was retained at the injection site for up to a period of three weeks and promoted a 4-fold increase in blood vessel formation compared with that of alginate or vehicle controls as confirmed by CD31 staining. Notably, as compared to alginate, Laponite gels did not release VEGF, indicating a strong interaction between the growth factor and the nanoclay and suggesting that Laponite enhancement of VEGF efficacy is due to its retention at the implantation site for a prolonged period. Our approach provides a robust method for the delivery of bioactive recombinant VEGF without the necessity for complex hydrogel or protein engineering.

In medicine, it is important to deliver drugs to a particular location in the body. Often, however, the drugs are quickly broken down and carried away in the blood before they can exert their effect. In this study, we used a type of synthetic clay, called Laponite$^{TM}$, to preserve a molecule, named VEGF, that stimulates the growth of blood vessels. Previously, we have been able to bind VEGF to the surface of clays, but the clay is not effective when injected or applied as a gel. Herein, we show that we can mix VEGF with the clay and that it strongly stimulates blood vessel growth. We speculate that this would be a useful material for skin wound healing.

Keywords: clay, nanosilicate, VEGF, nanoclay, angiogenesis, growth factors.

## Introduction

Preservation and retention of the activity of therapeutic proteins at the site of delivery are major goals in regenerative medicine. Growth factors such as vascular endothelial growth factors (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) have significant potential in promoting the growth and regeneration of cells and tissues [1]. However, proteins are intrinsically labile and are often not long-lived enough to ensure significant biological effect [2]. This may be compounded at sites requiring regeneration, which are often characterised by inflammation and high local concentrations of proteases. Furthermore, local injection of soluble growth factors may result in only transient activity before systemic clearance through the lymphatic and circulatory systems [3]. For these reasons, it is likely that for their efficacy, growth factors require a carrier that stabilises and protects them at the site of interest.

VEGFs comprise a family of growth factors that are responsible for increasing vascular permeability, endothelial cell signalling and proliferation to stimulate angiogenesis [4,5]. Recombinant VEGF proteins, most notably the predominant form of VEGF-A, $VEGF_{165}$, have been investigated for more than 20 years as a therapy for promoting angiogenesis in degenerative diseases or injuries [6], for example, stroke, bone fracture and skin wounds. However, their use is limited by the high doses required [7], subsequent off-target effects such as hypotension [8] and suboptimal angiogenesis causing, for example, severe vascular leakage and subsequent oedema [9].

To overcome these challenges, VEGFs can be incorporated within solid scaffolds, either naturally derived or synthetic, to control their release. Early studies used alginate as a depot to deliver VEGF in its active form to cultured cells [10]. This seaweed-derived polysaccharide material provides a relatively inert carrier scaffold that can be gelled in situ using a divalent cation such as $Ca^{2+}$ but which does not significantly restrict protein

diffusion. To better control VEGF release, the physical properties of the scaffold could be modified – for example, by controlling the surface area-to-volume ratio of polymer microparticles [11]. Alternatively, growth factors have been covalently crosslinked to the scaffold 'backbone'. Zisch *et al.* demonstrated enhanced angiogenesis when VEGF was covalently crosslinked with donor-derived fibrin [12]. Some biomaterial strategies have taken advantage of the affinity of heparin for VEGF by incorporating heparin within the backbone of the scaffold, thereby promoting VEGF retention [13–15], while VEGF has also been covalently linked directly to the backbone of polymeric hydrogels (based on PEG [16]). An advantage of these biomaterials is that other peptide motifs can be engineered into the polymeric backbone, for example, MMP-sensitive peptides or cell-binding motifs such as peptides containing the RGD amino acid motif. Together, this allows concurrent cell attachment ingrowth, degradation and growth factor release. These biomaterials have found application in a wide range of preclinical models such as in cardiac repair [17] and stroke [18], but their translation to the clinic is limited by their complexity. There remains a pressing need for simple biomaterials that better localise and preserve growth factor activity.

We have investigated the notion that inorganic, clay nanomaterials may overcome these shortcomings. Clays are inorganic, layered particulate materials that occur as a natural product of the weathering of rock or as a product of controlled hydrothermal synthesis from inorganic salts. When mixed with water, clays form plastic or viscoelastic pastes or gels [19]. Clays are highly sorptive for biomolecules, a property that has been used for many years, including as orally taken gastrointestinal protectors [20], skin exfoliation agents [21,22] and blood clotting agents [23]. More recently, we [24,25] and others [26,27] have tested the idea that synthetic nanoclays, such as Laponite$^{TM}$ XLG

($Na^{+}_{0.7}[(Mg_{5.5}Li_{0.3}Si_8O_{20}(OH)_{4)}]^{-}_{0.7,}$ hereafter referred to as 'Laponite') may be suitable as delivery agents of proteins for regenerative medicine.

Like many clays, Laponite has a long history of use in the food and cosmetic industries, suggesting that it is nontoxic and well tolerated, even at high doses. Aqueous dispersions of Laponite-mineral nanoplatelets (25 nm in diameter and ~1 nm in thickness) display self-assembly properties because of their charge anisotropy and ability to form thixotropic (shear-thinning), clear, colloidal gels [28]. These properties allow injection of Laponite through a hypodermic needle, resulting in the spontaneous formation of irreversible gels on contact with blood proteins and ions through a diffusion gelation mechanism, without the need for further chemical modification [29]. These properties make clays extremely attractive as, for example, tissue fillers for regenerative medicine, or in dressings or salves on the skin surface.

In this study, we tested the hypothesis that the activity of $VEGF_{165}$ could be preserved and sustain an angiogenic response following incorporation within the bulk of injected Laponite gels.

## Methods

### *Biomaterial preparation*

Three per cent (w/v) suspensions of Laponite XLG (BYK Additives, Widnes, UK) were prepared by slowly adding Laponite powder to distilled water under rapid agitation. Suspensions were autoclaved and volume adjusted with sterile $ddH_2O$. Alginate solutions (1.1% (w/v)) were prepared from anhydrous ultrapure alginate (NovaMatrix, Sandvika, Norway), with UV sterilisation for 30-60 minutes before preparation. To crosslink the alginate gel suspensions, $CaCl_2$ was added at a final concentration of 100 mM.

***Human umbilical vein endothelial cell (HUVEC) isolation and culture***

HUVECs were isolated from umbilical cords collected from Princess Anne Hospital, Southampton, UK, with the approval of Southampton and South West Hampshire Local Research Ethics Committee (Ref: 05/Q1702/102). HUVECs were isolated and cultured as described by Dawson *et al* [24], with some minor modifications. Briefly, the umbilical cord was cut at both ends; to one end, a cannula was inserted into an exposed vein and secured with ties. Sterile PBS was first flushed through the cord until the waste PBS collected at the other end was clear. The umbilical cord was then clamped at one end, and sufficient 5 mg/ml collagenase B (Sigma-Aldrich, Poole, UK) was added to fill the vein before incubation at room temperature for 1 hour. The collagenase B solution was then removed and centrifuged at 200 *g* for 5 minutes, and the supernatant was discarded. Cells were resuspended in endothelial cell growth medium (ECGM), which consisted of Medium 199 (Lonza), 10% foetal bovine serum (FBS; Life Technologies, Paisley, UK; product: 10270106, batch: 41Q4297P), penicillin 100 U/ml–streptomycin 100 U/ml (Sigma-Aldrich/Merck) and endothelial cell growth supplement (PromoCell GmbH, Heidelberg, Germany). The re-suspended cells were cultured at 37°C/5% $CO_2$ under humidified conditions. Following incubation, cells were sub-cultured using ECGM and passages of 1-4 were used in all experiments.

***Release of VEGF by Laponite/alginate hydrogels***

To assess the release of VEGF from biomaterials, VEGF was premixed with Laponite or alginate at a concentration of 40 µg/ml. Ten-microliter aliquots of biomaterial-VEGF were transferred into low-protein-binding microcentrifuge tubes (Eppendorf®, LoBind) containing 90 µl assay diluent. Biomaterials containing no VEGF and media with

aqueous VEGF added served as negative and positive controls, respectively. The medium was incubated at 37°C/5% $CO_2$ under humidified conditions and then recovered at various time points up to 3 weeks and stored at -20 ℃ until required for protein analysis.

To analyse the protein content in the supernatant recovered from biomaterial tubes, an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, UK) was performed according to the manufacturer's instructions.

***HUVEC 2D tubule formation on Laponite gels***

To promote cell attachment and growth on Laponite gels, human fibronectin (Merck Millipore, Watford, UK) was premixed with Laponite gels to a final concentration of 50 µg/mL [24]. Cell culture substrates were formed by adding 150 µl of Laponite to the bases of wells of 24-well plates, followed by briefly centrifuging to ensure an even Laponite layer and incubating for 1 hour at 37°C for gelation.

To test the activity of $VEGF_{165}$ (PeproTech, London, UK; hereafter denoted as VEGF) in promoting tubule formation when associated with the Laponite biomaterials, VEGF was either mixed or surface adsorbed to pre-formed Laponite gels. For the former, reconstituted human recombinant VEGF was added to final concentrations of 1 to 5 µg/ml and mixed by vortexing before forming substrates. For the latter, VEGF in ECGM was incubated at a concentration of 12.6 ng/cm$^2$ at room temperature for one hour.

Following substrate formation, HUVECs were resuspended in ECGM containing 10% FBS and 40 ng/ml basic fibroblast growth factor (bFGF; Thermo Fisher Scientific-

Invitrogen, UK) and seeded at a density of 5 x $10^4$ cells/well (n=6). To test the activity of soluble growth factor, VEGF was added to the medium at concentrations of 0–40 ng/mL.

***Image analysis of HUVEC 2D tubule network***

A freely available macro for ImageJ named 'Angiogenesis Analyzer' by Gilles Carpentier (Gilles Carpentier. Contribution: Angiogenesis Analyzer, ImageJ News, 5 October 2012, http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ#nb1) for the NIH Image J 1.47v program was used to automatically quantify the 2D tubule network of HUVECs. Please refer to Supplementary Information 1 [S1] for more information regarding software configuration settings.

***Animals***

Eighteen male MF1 mice (Biomedical Research Facility, University of Southampton) aged between 8 and 10 weeks old were used to investigate the localisation of VEGF using Laponite hydrogels. All animals were bred and maintained at Biomedical Research Facility, Southampton, in a temperature-controlled environment (20°C-22°C) with a 12 h light/dark cycle (lights on at 0600 hrs). Food and water were provided ad libitum both pre- and post-injection. All procedures were performed under ethical approval, obtained under project licence PPL 30/2971.

***Subcutaneous VEGF-biomaterial treatments***

Mice were anesthetised using an inhalant anaesthetic (isoflurane; product from Centaur, Castle Cary, UK), and the whole dorsal region was shaved. On the left side of the dorsum, pre-prepared Laponite gels were administered subcutaneously by injection (50

μL) at three separate locations (rostral to caudal) with three different VEGF doses (1, 10 and 40 μg/mL VEGF) (n = 6 mice). On the contralateral side, 3 vehicle Laponite treatments were administered (negative control). A control biomaterial hydrogel, alginate, was administered similarly (n = 3 mice). For the preparation of alginate for injection, $CaCl_2$ was added at a 1:10 dilution from a 100 mM stock solution (final concentration of 10 mM) to the alginate/VEGF solution to a final concentration (of alginate) of 1.1% (w/v). These gels were then immediately drawn into 1 mL syringes before placement of the needle onto the syringe nozzle and extrusion subcutaneously. Note that no fibronectin was added in these experiments. Mice were killed by $CO_2$ asphyxiation for 3-5 minutes, followed by cervical dislocation. The biomaterial along with the surrounding cutaneous tissue was surgically removed after 21 days and fixed in 4% (w/v) paraformaldehyde (PFA) for 18 hours. Tissue samples were then transferred to 70% ethanol and stored at 4°C until required for processing.

***Unbiased macroscopic angiogenic scoring***

Upon tissue harvest, photographs of each biomaterial treatment and dose were captured using a Nikon D3200 digital single-lens reflex (SLR) camera. A scaled ruler was present for all images captured. All images were then labelled (biomaterial treatment, VEGF concentration, time point). Using the Measure module within ImageJ, the assigned label was ‘measured’ using the Batch command to generate a list of all image names with a corresponding number. These data were imported into Microsoft Excel (2016 version). Within Excel, a column was inserted adjacent to the label data set. In the first cell of this column, the =RAND() command was entered, and it was then copied into every cell; this command created a list of random numbers, which then allowed the label data set to be sorted according to this random list. The images were then arranged

on a blank page (no label included). The sheets that contained these randomised images were used as the basis for a randomised blinded questionnaire used to measure the degree to which angiogenesis had occurred. Please refer to Supplementary Information 2 [S2] for a copy of the questionnaire that was designed for this study.

***Histological analysis***

Fixed samples were washed in PBS before incubation with 30% (w/v) sucrose (Sigma-Aldrich/Merck) in PBS overnight at 4°C. The samples were then briefly washed with an optimal cutting temperature (OCT) compound (CellPath, Newtown, UK) to remove excess sucrose and then immersed with the OCT compound in a cryo-mould. Cryo-moulds were placed into a solution of pre-cooled (-80°C) isopropanol (dry ice was added to isopropanol) to allow controlled sample freezing. Moulds were stored at -80°C until required for sectioning. Sequential sections were cut at a 10 µm thickness using a cryotome (maintained at -25°C to -30°C) and mounted on charged glass slides and placed on a warming rack (37°C) for 30 minutes and then stored at -80°C. When required for histological and immunohistological staining, cryo-sections were thawed at room temperature for 10 minutes and washed in PBS for 10 minutes.



***Histochemistry***

*Anti-CD31 immunohistochemistry.* A rat detection kit for anti-mouse CD31 (MenaPath, Menarini Diagnostics, Winnersh, UK; product: MP-517-RTK6) was used in combination with an anti-CD31 antibody (rat monoclonal, MenaPath, Menarini Diagnostics, product: MP-303-CM01) following the manufacturer's instructions. Slides were dehydrated and mounted with cover slips using the DPX mounting medium (Thermo Fisher Scientific; product: 10050080).

*H&E.* Following OCT removal, slides were stained with hematoxylin and eosin before mounting with coverslips.

*Auramine O.* Slides were flooded with pre-prepared Auramine O (Auramine O 0.3 g, phenol 3.0 g, distilled water 100 ml) (Sigma-Aldrich/Merck) for 15 minutes at room temperature in the dark. Excess Auramine O solution was discarded, and slides were washed with distilled water (2 x 2-minute washes). Slides were immediately mounted with coverslips using Fluoromount™ (Sigma-Aldrich/Merck; product: F4680-25ML) and sealed with nail varnish. Slides were stored in the dark and imaged within 3 days.

***Microscopy***

Histological sections were imaged using the Olympus BX 51 dotSlide virtual slide microscope system (Olympus Life Science, Southend-on-Sea, UK). The captured images were extracted and analysed using Fuji ImageJ v1.50b, BIOP PT VSI plugin and Olympus Virtual Slide Desktop 2.4 software.

***Analysis of CD31 staining by Chalkley count***

The Chalkley point-overlap morphometric technique (more simply referred to as 'Chalkley method') is a relative area estimate method to measure the abundance of microvessels in a immunohistochemical sample [30]. Using a similar approach documented in a previous publication [31], a digital Chalkley grid overlay was applied to a 20X magnification image at 3-5 different 'hot-spot' regions. Counts of these 3-5 regions that landed on the most positively stained structures through rotating the digital overlay were recorded and the mean values used for analysis.

***Analysis of cellularity***

H&E-stained image samples were imported into a free open-source software program, called Orbit Image Analysis (revision 2.67; http://www.orbit.bio/download/). This software was used to isolate/segment H&E-stained (nucleated) cells and measure and compare the % area of pixels. Please refer to Supplementary Information 3 [S3] for software configuration settings.

***Statistical analysis***

One-way or two-way analysis of variance (ANOVA) was used with post-hoc Tukey's multiple comparison tests to determine individual p values between groups, as indicated in figure legends. Statistical significance was defined at $p < 0.05$.

**Results**

*VEGF is sequestered tightly by Laponite gels and not released*

To test the release profile of VEGF premixed within an injectable Laponite gel formulation, we performed an *in vitro* release assay using an ELISA for VEGF. For a period of 21 days, no VEGF was released from Laponite-VEGF gels (incorporated at a concentration of 40 μg/mL). In direct contrast, VEGF was released rapidly from alginate-VEGF gels, with 43.1 ± 4.3% released at 12 hours and 77.4 ± 6.6% at 24 hours (Figure 1A).

*Localisation of VEGF encapsulated by Laponite stimulates angiogenesis in vitro*

VEGF has previously been shown to retain bioactivity following surface adsorption to Laponite nanoclay from aqueous media [24]. Its utility would be substantially increased, however, if it could be delivered stably within the bulk of a gel or paste, for example, as an injectable or topically applied material. To explore the potential of this approach, we

first tested whether the activity of VEGF was preserved following incorporation into the bulk of Laponite gels as compared to that of aqueous VEGF. HUVECs, which form quantifiable tubule networks in response to VEGF, were cultured on the surface of 3% (w/v) Laponite gels pre-mixed by vortexing with VEGF at concentrations between 0 and 5.00 μg/ml. For comparison, cells were cultured on the surface of 3% Laponite gels (premixed with vehicle alone; no VEGF) in the presence of aqueous VEGF at concentrations of 0–40 ng/ml (a schematic of the experimental design is shown in Figure 1B).

Aqueous VEGF promoted HUVEC tubule formation on Laponite substrates in a dose-dependent manner, with a maximal effect at a concentration of 0.04 μg/mL and a half-maximal effect at ~0.01 μg/mL (Figure 1C & D). VEGF adsorbed at a concentration of 12 ng/cm$^2$ also promoted maximal tubule formation (Supplementary Figure 1) as demonstrated in a previous publication [32]. When VEGF was mixed in the bulk of the Laponite, it retained activity. However, a concentration of 5 μg/mL VEGF was required to exhibit the equivalent stimulatory effect as seen with aqueous VEGF and Laponite-adsorbed groups, with a half maximal response at ~1 μg/mL ($p = <0.01$) (Figure 1C & D). Together, these data indicate that VEGF activity is preserved following mixing in the bulk of the Laponite, similar to surface adsorption but that mixing reduces either bioavailability or protein activity.

*Laponite-incorporated VEGF stimulates and supports blood vessel infiltration*

We next tested whether VEGF mixed in 3% (w/v) Laponite (hereafter referred to as VEGF-Laponite) could induce angiogenesis *in vivo*. VEGF-Laponite was injected subcutaneously on the dorsum of male aged-matched mice (Figure 2A). On the basis of

our *in vitro* observations, we chose a range of VEGF concentrations between 1 and 40 μg/ml (total doses of between 0.05 μg and 2 μg). Alginate, a well-studied biomaterial used in drug delivery [33], was used as a control hydrogel at a concentration of 1.1% (w/v) containing the same range of VEGF concentrations.

Laponite and alginate biomaterials could be observed macroscopically at injection sites for at least 21 days, indicating retention and cohesion of complete gels (Figure 2B). At tissue harvest, blood vessel infiltration of VEGF-Laponite gels at concentrations of 10 or 40 μg/mL was evident (Figure 2B). At 40 μg/mL, blood vessels from the surrounding tissue were observed growing into and along the surface of the implanted VEGF-Laponite. In some cases, extensive vessel branching was present (Figure 2B lower panel; arrows). In contrast, alginate-VEGF hydrogels exhibited less overt ingrowth. Even when 40 μg/mL VEGF was incorporated, pre-existing blood vessels present at the periphery of the alginate implant (Figure 2B lower panel) showed little evidence of branching into the biomaterial.

To quantify the degree of blood vessel infiltration, blinded observers scored images of samples on a scale of 0–6 (see Supplementary Information 2 questionnaire for details of scoring criteria). The score for blood vessel infiltration was significantly greater in Laponite gels delivered at concentrations of ≥10 μg/mL than in those delivered at concentrations of ≤1 μg/mL ($n = 6$, $p < 0.001$) (Figure 2C). In contrast, no significant increase in blood vessel infiltration was observed in alginate-VEGF gels at any concentration. The increase in vessel infiltration was time-dependent; there were no significant changes in blood vessel infiltration for any gel at d14, with increases becoming evident only at d21 (Figure 2D). These data indicate that, in contrast to

alginate, Laponite preserves the activity of VEGF *in vivo*, either by preventing release of the growth factor or by preserving its activity.

*Laponite-incorporated VEGF stimulates angiogenesis and cell invasion in vivo*

Next, we investigated cell infiltration and angiogenesis in implanted VEGF-containing Laponite and alginate gels. The harvested biomaterial tissue samples were sectioned and histological staining performed. Staining of tissue sections with anti-CD31 (an endothelial cell marker) showed greater density of blood vessels at higher (10 and 40 µg/ml) VEGF concentrations when localised by Laponite gels; in contrast, negligible CD31 was apparent in the alginate biomaterial treatment (Figure 3A). Quantification of CD31+ blood vessels was performed using a Chalkley grid analysis (Figure 3B), which confirmed an increase in the number of vessels with increasing VEGF concentration in Laponite samples (however, only 40 µg/ml VEGF showed significant difference when compared with other concentrations and Laponite vehicle ($p$ = <0.01)) (Figure 3C). In summary, these data indicate that the injectable Laponite gel facilitates the ingress of cells and blood vessels and that this is promoted by the inclusion of VEGF in the bulk of the gel.

In parallel with increases in blood vessel infiltration in Laponite gels, haematoxylin and eosin (H&E) staining of day 21 Laponite and alginate samples containing 40 µg/mL VEGF showed differences in cell invasion. Laponite samples appeared rich in cellular content with regions of biomaterial still present, whereas alginate samples contained large open spaces devoid of cells (Figure 4A). Auramine O, a substituted diphenyl-methane cationic dye, was used to label implanted nanoclay against biological tissue on the basis of a dramatic increase in the fluorescence emission upon stabilisation by clay

nanoparticles [34,35]. A substantial volume of nanoclay remained at 21 days but was fragmented, with cells and tissue present throughout the fragments (Figure 4A).

Analysis of H&E-stained images involved software-based segmentation of haematoxylin hotspots to identify cell nuclei and determine gross cellular infiltration (Figure 4B). Image analysis showed that there was a trend of greater cellularity in Laponite samples with regard to increasing VEGF concentrations, with significantly greater cell infiltration at 40 μg/ml (Figure 4C, $p = < 0.05$). In contrast, there was no significant difference in cell infiltration for alginate gels irrespective of VEGF concentration (Figure 4C, 0 vs. 40 μg/mL alginate, $p = 0.14$).

## Discussion

This study demonstrates the utility of a robust, well-defined biomaterial in sequestering and preserving the activity of VEGF for prolonged periods of time *in vivo*. We showed that VEGF can be stably incorporated into the injectable Laponite nanoclay gels, that the VEGF was active both *in vitro* and *in vivo* and that the material acted as a stable template for angiogenesis and cell infiltration.

A medium concentration of 40 ng/ml of VEGF was necessary to promote maximum tubulogenesis in HUVECs cultured on Laponite substrates. This concentration is equivalent to the *total* VEGF required to elicit maximal tubulogenesis when pre-adsorbed to the Laponite surfaces (i.e., preincubation of Laponite substrates with 300 μL of 40 ng/ml medium (equal to 12 ng/cm$^2$) before culture of cells on the surface resulted in the *same* degree of tubulogenesis as that when 40 ng/ml was included in the medium concurrent with cell growth (Supplementary information 4 [S4] and [24]). This

indicates that Laponite surface binding/interaction does not reduce the bioactivity of VEGF.

Relatively higher concentrations of VEGF were required to promote maximal tubulogenesis in HUVECs when delivered premixed in Laponite. On first consideration, this might suggest that mixing reduces the activity of VEGF. While we cannot entirely exclude some loss of activity through mixing, it is important to recognise the qualitative differences in the approaches. In our study, 150 μL of Laponite gel was required to ensure a reproducible coating of each well (1 $cm^2$ in area), equivalent to a mean depth/thickness of 1500 μm. By simple arithmetic, it can be calculated that, at 5 μg/ml, the uppermost 24 μm of the 'cylinder' of Laponite would contain 12 ng VEGF per $cm^2$ — the density of VEGF necessary for maximal tubulogenesis. First, the approximate diameter of endothelial cells was 13-25 μm [36–38], second, there was lack of any significant penetration of cells into the Laponite substrate *in vitro* and third, Laponite gels tightly sequestered VEGF (Figure 1A). Therefore, we consider it very likely that the majority of VEGF in this 'bulk mixed' configuration is sequestered away from the cells, and therefore, it is not bioavailable during the tubulogenesis assay.

Our *in vivo* findings suggest a localised, prolonged angiogenic effect of VEGF when sequestered in Laponite as compared to that in an alginate carrier that facilitates burst release, and these findings have intriguing corollaries in drug delivery. Conventional hydrogel drug delivery strategies tend to target controlled, sustained release from the biomaterial with time as a necessary strategy for recruiting local endogenous cell populations [27]. Conversely, our data suggest that a sustained localised concentration of VEGF with rather minimal release is effective for promoting strong local angiogenesis. This observation is notable for two reasons. First, the data imply that Laponite gels can host and even promote the invasion of responsive populations into the

gel structure before a VEGF-mediated response. Second, the activity of the sequestered VEGF is sustained in the gel before becoming bioavailable to invading cell populations.

VEGF has been shown to have a half-life of ≤90 minutes *in vitro* [39] and ~34 minutes *in vivo* [40]. The observation of significant blood vessel formation from a single dose of VEGF 21 days after administration (a clear increase was apparent only between days 14 and 21) implies either that the signalling important for subsequent vessel formation occurs at very early time points or that the activity of VEGF is preserved and prolonged by incorporation in Laponite. Considering the first point, given a conservative estimation of a ~90-minute half-life for VEGF (which is its known *in vitro* half-life in buffer at room temperature [39]), the quantity of active protein remaining from a 2 μg dose after only 24 hours would be at a sub-picogram range, whereas ng/ml concentrations are required for receptor activation *in vitro* [41,42]. Furthermore, cell invasion is likely to require time on the scale of days rather than hours, and therefore, at early time points (<24 hours), initial presentation of VEGF would be limited to the biomaterial surface. Supporting this, at 21 days, penetration of Laponite by cells was incomplete, with cell-free areas visible in the central regions of the nanoclay, suggesting gradual cell invasion. In addition, after implantation, cell invasion is likely to be initially mediated primarily by immune cells such as neutrophils and macrophages rather than the target cell population responsible for blood vessel formation (endothelial cells) as seen in other reports of biomaterial implantation [43,44]

Such considerations are underscored by the requirement in previous studies for much higher doses of soluble VEGF to be necessary to evoke a biological response and by data indicating that VEGF half-life can be extended by immobilisation in scaffolds. For example, a study conducted by Galiano *et al.* on wound healing [45] showed that it was necessary to add 20 μg of VEGF topically to each wound every other day for 10 days to

exhibit a biological effect (a dose of 100 μg in total, compared with 2 μg total dose in the data presented in the current study). Furthermore, it was observed that locally applied VEGF induced undesired angiogenic responses at sites distant from the site of application, again suggesting that both protein degradation and protein diffusion are important factors for sustained, local activities. Other studies have sought to localise VEGF by passively binding it to scaffolds such as fibrin and have shown that, when coupled in this manner, VEGF can retain activity for periods of up to 2 weeks [46], again supporting the notion that Laponite may also stabilise VEGF (although perhaps by a different mechanism). There remains a paucity of data on how VEGF protein activity is stabilised by binding to other proteins or to biomaterials, but this remains an important area for future investigation. In view of these observations, we consider it likely that Laponite preserves the bioactivity of VEGF for a prolonged time *in vivo*.

Our approach has some limitations. For example, although we were able to measure the degree of blood vessel infiltration quantitatively by macroscopic and microscopic means in the presence of VEGF, we did not address the morphology of the vessels. It has been shown previously that sustained and high levels of $VEGF_{165}$ can cause formation of dysfunctional blood vessels with irregular and leaky lumina [47–49]. Others suggest that this may be due to subtle fluctuations in microenvironmental VEGF concentration [48]. We did not test directly the homogeneity of VEGF dispersion throughout the Laponite, but future studies may address this directly. Because of the highly sorptive nature of Laponite, we consider it likely that tight binding to nanoclay particles should provide precise control over microenvironmental concentration, however. Finally, as we recently reported, Laponite biomaterials have limited porosity, with sub-micron pore sizes in the native state (in aqueous solution) enlarging to a maximum of ~2 μm when in contact with serum [29]. The fact that the majority of blood vessels and cell invasion

were found in the peripheral regions of injected gels may suggest that porosity limits the ingress of new tissue into the gels. Future approaches may benefit from engineering porosity into Laponite, as has been achieved with other hydrogels such as by the production of gel microparticles, as has been done using gelatin [50] or by 3D printing constructs with an engineered microstructure [51].

In contrast to other approaches mentioned in the literature (which often rely on complex bio-engineering of either the matrices from which growth factors are delivered [52] or the growth factors [53], or a combination of both [54]), our approach allows effective localisation of an active growth factor in an injectable hydrogel without complex chemical crosslinking methods. As it is known that clay biomaterials also preserve the activity of another growth factor, bone morphogenetic protein 2 (BMP2) [25], exploration of nanoclay for the delivery of a range of therapeutic proteins may be a rich area for future research.



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**Author contributions**

DJT designed and performed experiments, analysed and interpreted data and contributed to writing the article; CEC contributed to the conception of the study, analysed and interpreted data and contributed to writing the article; RM analysed and interpreted data and contributed to writing the article; NAK performed experiments; JID contributed to the conception of the study, analysed and interpreted data and contributed to writing the article; NDE contributed to the conception of the study, performed experiments, analysed and interpreted data and contributed to writing the article. All authors approved the manuscript.

## Data availability

At the time of submission, the raw/processed data required to reproduce these findings could not be shared, as these data also form part of an ongoing study. The data would be made available on the acceptance of the article.



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**Figure Legends**

**Figure 1.** *Localisation of VEGF encapsulated by Laponite hydrogels stimulates in vivo angiogenesis.* (A) VEGF was not released from Laponite gels for a period of 21 days (red plot). This result is in contrast with alginate gels, where ~75% of the incorporated VEGF was rapidly released for a period of 24 hours. *** $p < 0.001$; **** $p < 0.0001$. n = 6; error bars indicate SEM. (B) Schematic illustrating 2D tubule formation of human vein endothelial cells (HUVECs) with Laponite hydrogels. The left side of the image shows a schematic of VEGF in media, and the right side of the image shows $VEGF_{165}$ incorporated in the Laponite gel (VEGF denoted as yellow stars). (C) VEGF stimulates tubule formation in HUVECs when cultured with VEGF alone in the medium (left) or when mixed with Laponite (right) as shown by phase-contrast microscopy with network formation analysis segmentation (scale bar = 500 µm). (D) Network formation in response to VEGF was dose-dependent, but a higher concentration was required when mixed with Laponite as compared to that when added to the medium. Error bars = SD, n = 3; one-way ANOVA was performed, with Tukey's post-hoc test applied for multiple comparisons; * $p < 0.05$, ** $p < 0.01$, ** $p < 0.001$, ****$p < 0.0001$.

**Figure 2**. *VEGF encapsulated by Laponite hydrogels stimulates in vivo angiogenesis*. (A) A schematic showing subcutaneous injection of Laponite or alginate on the dorsum of healthy mice at a range of concentrations. A vehicle control was administered on the contralateral side. (B) Increased angiogenesis was visible in the macroscopic images at biomaterial sites that contained high VEGF (>1 µg/ml) concentrations. Blood vessels were observed infiltrating the Laponite and branching over the surface (blue arrows indicate vessel branching, and white arrows indicate vessels located at the biomaterial periphery) in VEGF-Laponite hydrogels. Scale bar = 5 mm. (C) Blinded scoring of harvested biomaterials confirmed that there was a significant increase in angiogenesis in

Laponite-VEGF-treated groups at VEGF concentrations of 10 and 40 µg/ml on day 21 as compared to that in alginate-VEGF-treated groups. (D) Differences in blood vessel infiltration became apparent only at day 21; no significant change in angiogenic score was seen on day 14. n = 3; Error bars = SD. Two-way ANOVA was performed with Tukey's post-hoc test applied for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, ** $p < 0.001$, ****$p < 0.0001$.

**Figure 3.** *Localisation of VEGF encapsulated by Laponite hydrogels stimulates in vivo angiogenesis; immunohistochemical analysis.* (A) The density of blood vessels was markedly greater in VEGF-Laponite than in Laponite alone, as indicated by anti-CD31 staining. This was not obvious in VEGF-alginate controls. Scale bar = 100 µm. (B) Blood vessel density was quantified using a Chalkley grid (see Methods); the upper panel is an example of a low Chalkley count (2 vessels identified), and the lower panel is an example of a high Chalkley count (7 vessels identified) (shown by red circles). (C) Chalkley count analysis showed that 40 µg/ml VEGF mixed with Laponite caused significant growth of blood vessels when compared with that at lower VEGF concentrations and all VEGF concentrations present within the alginate biomaterial. Error bars = SD. One-way ANOVA was performed with Tukey's post-hoc test applied for multiple comparisons. *, **, *** and **** denote that staining of blood vessel marker, anti-CD31, shows a significant increase ($p = <0.05, <0.01, <0.001$ and $<0.0001$, respectively).

**Figure 4.** *Cells invade and form blood vessels within Laponite in vivo* (A) Haematoxylin and eosin and Auramine O revealed pronounced cellular invasion in subcutaneously implanted Laponite gels, with minimal cell invasion in alginate scaffolds. Left panel scale bar = 1 mm; right panel scale bar = 200 µm. Arrows indicate skin epithelium; * indicates residual nanoclay gel. (B) Example image of high-powered magnification of cells stained with haematoxylin and eosin (left panel); using computer-based software, cell invasion into the biomaterial was identified, cells were segmented and the density of cells was measured as percentage cell coverage. Scale = 100 µm. (C) Segmented cell analysis showed greater cell invasion in Laponite-treated groups when incorporated with increasing VEGF concentrations; in contrast, alginate treatments showed very minor changes. Quantification of cell coverage confirmed this trend: 40 µg/ml VEGF mixed within Laponite exhibited significant difference, but no trend was observed for alginate. Error bars = SD. One-way ANOVA was performed with Tukey's post-hoc test applied for multiple comparisons. * denotes $p = <0.05$.

**FIGURE 1**

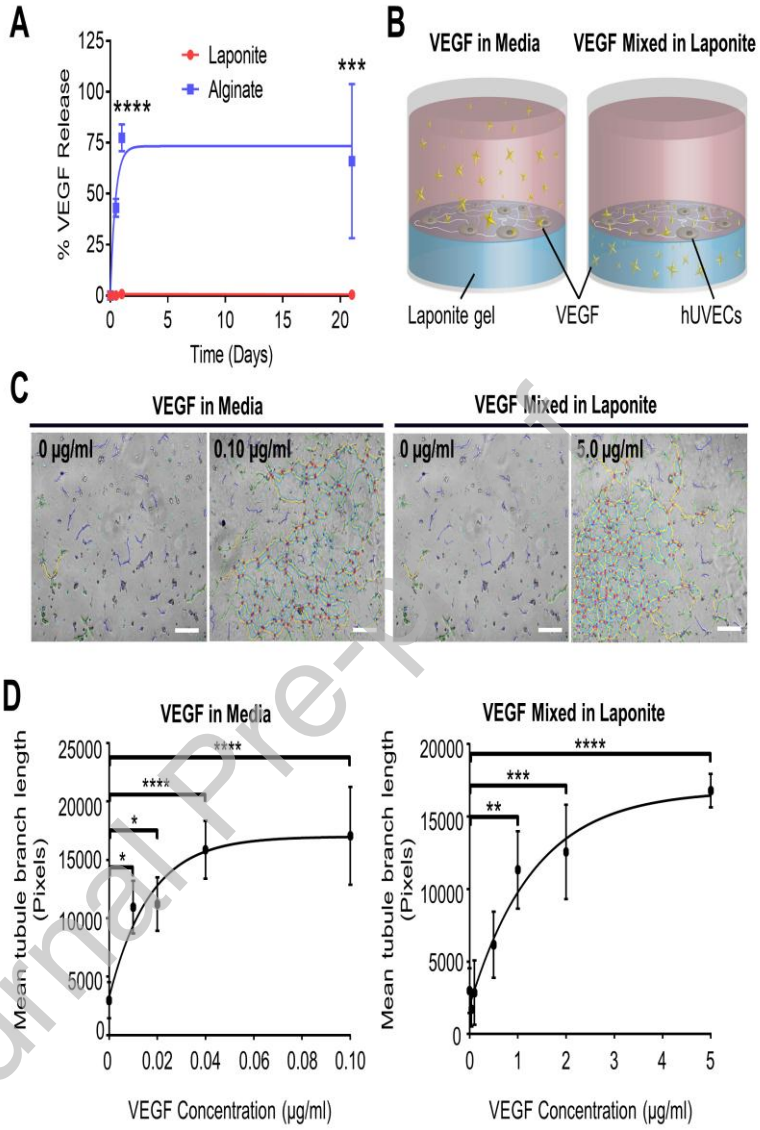


FIGURE 2

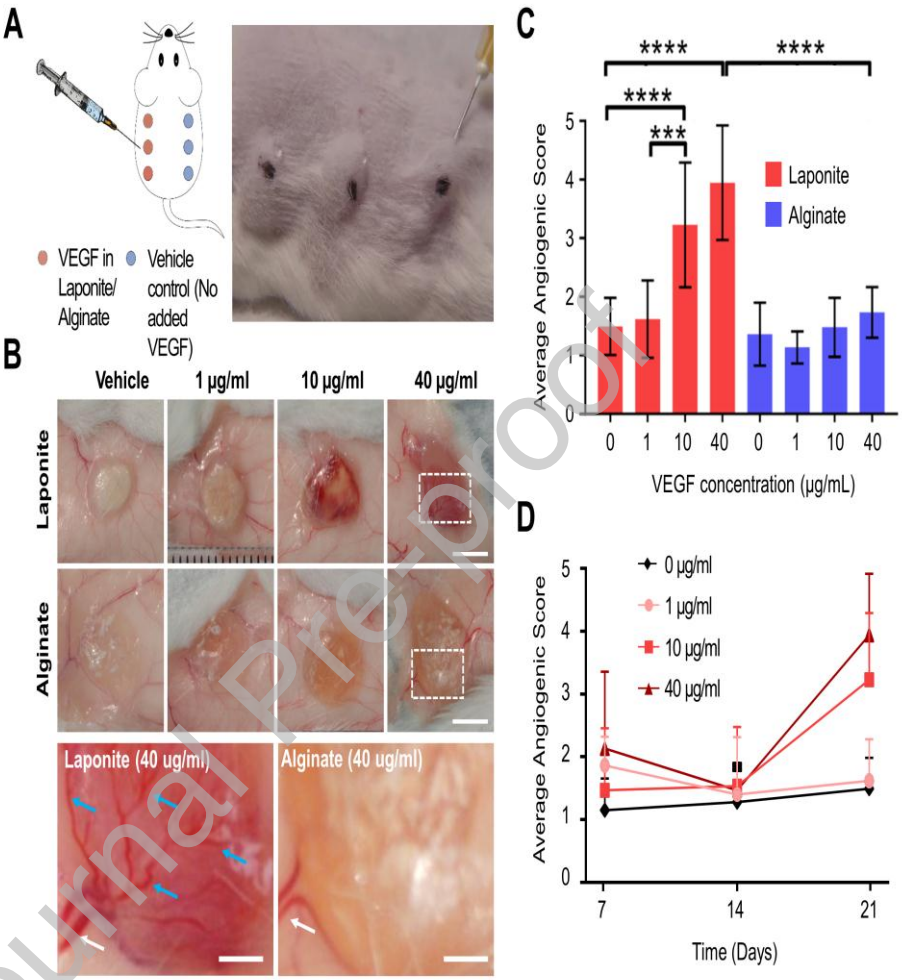


**FIGURE 3**

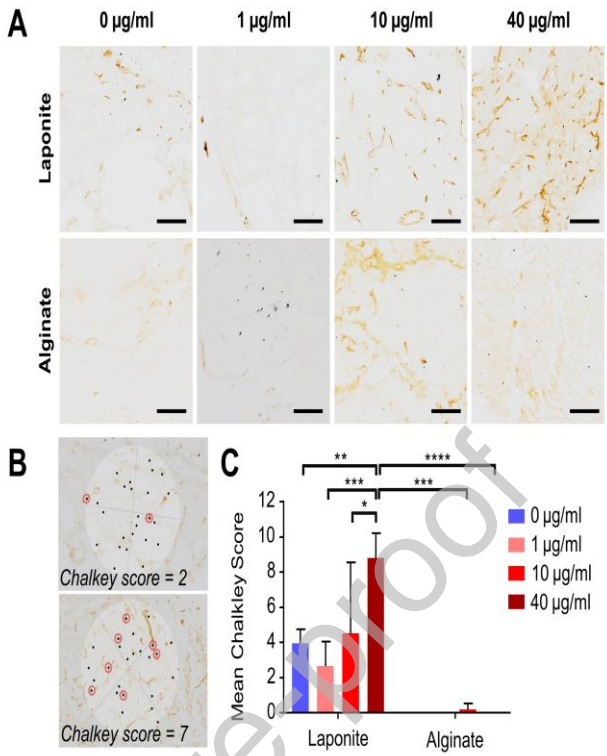
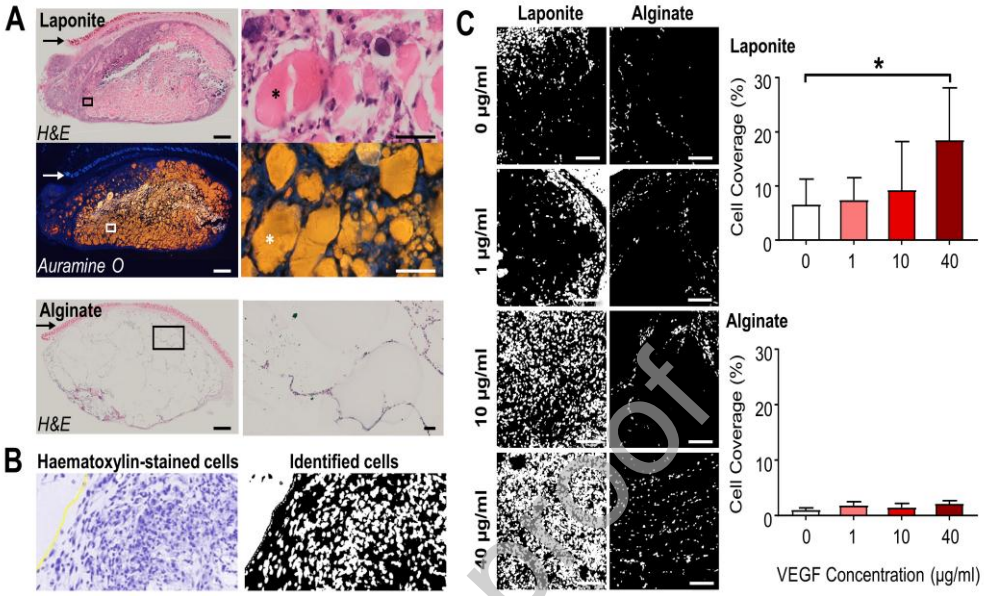


**FIGURE 4**



Graphical Abstract