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Novel Aptamer-based Implantable Scaffolds for Tissue Regeneration

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SUMMARY

Aims: The aim of the present study was to investigate whether immobilised anti-Fibronectin aptamers could selectively enrich biomaterial surfaces for Fibronectin and promote the attachment and growth of osteoblastic cells.

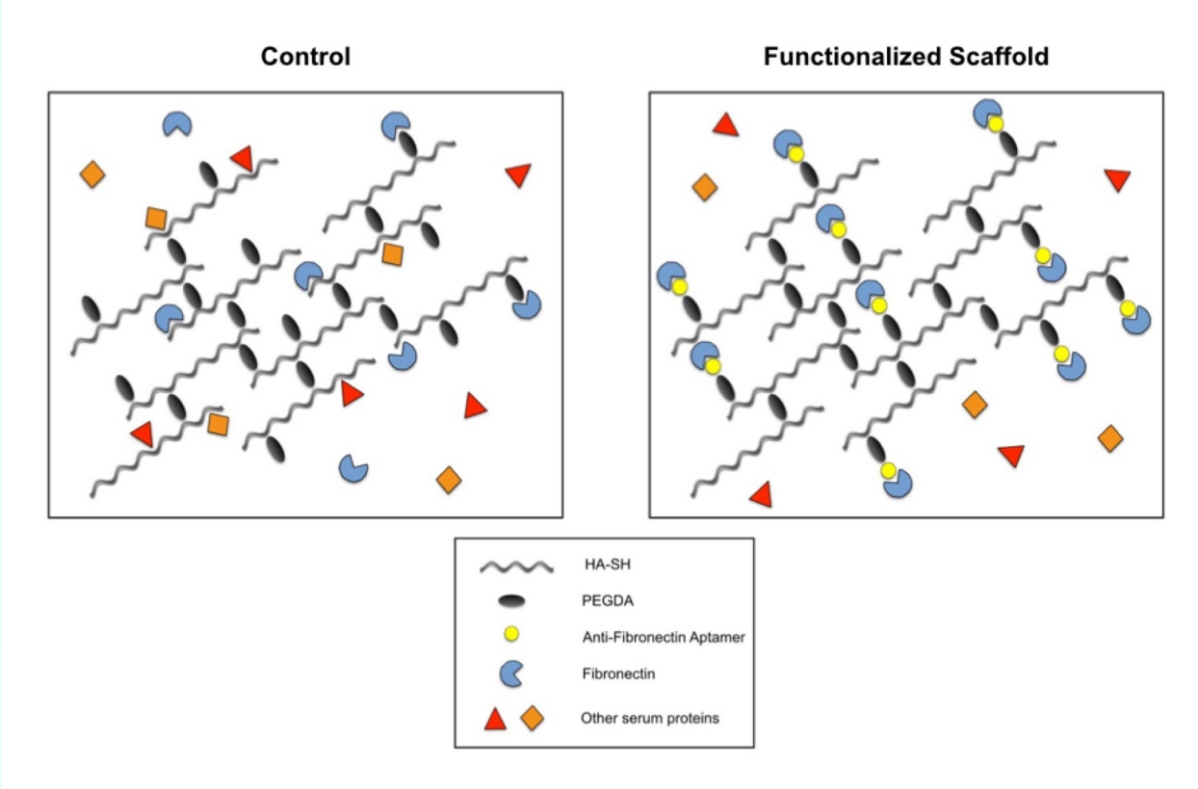
Methods: Polyethyleneglycole diacrylate/thiolated Hyaluronic Acid hydrogels (PEGDA/tHA) were coated with Anti human Fibronectin DNA aptamers. Human osteoblasts (hOB) were cultured on hydrogels for 10 days, rinsed with PBS and fixed for microscopy. Cells were also stained with anti-Vinculin antibody, TRITC-Phalloidine and DAPI for fluorescent labelling of focal adhesions, microfilaments and nuclei respectively. Hydrogels were then implanted in sub-cute pouches in rats and harvested 4 and 7 days after surgery for histology.

Results: Aptamers specifically bound to Fibronectin and enriched hydrogel scaffolds for this protein. Aptamers increased the number of adherent hOB cells on hydrogels after 10 days of culture. When stained with fluorescent markers, more cells were observed on aptamer-enriched hydrogels and their cytoplasm appeared more spread and richer in adhesion complexes than on control hydrogels. In vivo preliminary data confirmed that aptamers promoted scaffold colonisation by neighbouring cells by day 7.

Conclusions: Our data demonstrate that anti-Fibronectin aptamers promote scaffold enrichment for this protein, thus improving cell adhesion and scaffold colonisation.

INTRODUCTION

The successful integration of implantable biomaterials requires their integration with the surrounding extracellular matrix (ECM), a complex network of proteins with structural and signalling properties (Gattazzo et al., 2014). Biomaterials, once inserted into the surgical wound, get in contact with blood and spontaneously adsorb plasma proteins, based on protein availability and conformation (Micksch et al., 2014; Vogler, 2012). The presence of adsorbed proteins triggers subsequent host reactions, such as blood clot formation, inflammation, cell attachment on biomaterials (Hiraguchi et al., 2014), which otherwise would not display specific binding motifs for cell adhesion apparatuses, as those present on ECM components (Clause and Barker, 2013).
A possible approach to enhance the biological activity of a biomaterial is to promote adsorption of bioactive molecules from the host, by means of receptors that can specifically bind to and enrich the biomaterial surface with biological molecules of interest to provide an endogenous stimulus to cell colonisation. To reach this goal in the present study, hydrogel scaffolds were coated with DNA aptamers (Figure 1).



Aptamers are small, single stranded biomolecules, typically oligonucleotides, less than one hundred residues long (Song et al., 2012; Tuerk and Gold, 1990), which specifically bind to a target molecule (Mascini et al., 2012). Fibronectin was chosen as target for the present study because it is a widely available protein in plasma, so it would readily be available after implant insertion in an hypothetical surgical scenario. Moreover Fibronectin can enhance cell adhesion (Chatakun et al., 2014), and has been proposed as a coating for implantable biomaterials (Kim et al., 2011; Pendegrass et al., 2012; Rapuano et al., 2012).

MATERIALS AND METHODS

Aptamer preparation - We had DNA aptamers (Base Pair Biotechnologies, Pearland, TX) screened for against human Fibronectin (Advanced Biomaterix, San Diego, CA, USA) and functionalised with a short carbon chain containing a S-S bond on their 3' end (IDT, Coralville, IA, USA).

Hydrogels - Aptamers or TE buffer (Sigma-Aldrich, St.Louis, MO, USA) were mixed to Polyethylene Glycol Diacrylate (PEGDA) at the final concentration of 600 nM and incubated for 30' prior to addition of thiolated Hyaluronic Acid (tHA, Glycosan, Biotime Inc., San Francisco, CA, USA) in a 1:1 ratio. Gels were allowed to set for 1 hour. The thiol groups on the 3' end of aptamers bound to PEGDA acrylate groups through a nucleofilic addition (Michael's addition) and thus immobilised the aptamers on the matrix. Hydrogels were conditioned with Dulbecco modified MEM (DMEM, PAA, GE Healthcare, Uppsala, Sweden), 10% Human Serum (Type AB, HS, Sigma-Aldrich), 1% Penicillin and Streptomycin (Penstrep, Sigma-Aldrich) and 1% Glutamine (Sigma-Aldrich) for 1 hour prior to cell culture, to allow for Fibronectin binding.

Hydrogels selectivity for Fibronectin – To investigate Fibronectin absorption on hydrogels, Bradford assay was performed. Hydrogel containing 50 µg/ml aptamers were incubated with Fibronectin at different concentrations (75µg/ml, 150µg/ml) for 2 days at 4°C. Aliquots of surnatant were collected and protein concentration in surnatants and gels was measured with Bradford (BIO-RAD Protein Assay, BIO-RAD).

Cell cultures - HOB1 cells were obtained from the American Type Culture Collection (LGC Standards S.R.L., Sesto S.Giovanni, MI, Italy). They were grown in complete Dulbecco modified MEM as described above. Cell assays were performed in 96-well plates or 8-well chamber slides (Nunc, ThermoFisher, Waltham, MA, USA) in DMEM supplemented with 10% HS.

Immunofluorescence - Fibronectin staining was performed with rabbit polyclonal anti- Fibronectin antibody (F3648, Sigma-Aldrich). Samples were then labelled with FITC-antirabbit IgG antibody (ab97048 Abcam, Cambridge, UK). For cell imaging, cells were stained with a FITC-conjugated rabbit monoclonal anti-vinculin antibody (FAK100, Chemicon, Temecula, CA, USA), TRITC-conjugated phalloidin (FAK100, Chemicon, Temecula, CA, USA) and DAPI (D1306, Molecular Probes, Invitrogen, Carlsbad, CA, USA). The samples were mounted using an antifade medium (P7481, Molecular Probes, Invitrogen, Carlsbad, CA, USA) and examined using a Nikon Eclipse 90i (Nikon, Tokyo, Japan) microscope.

MTT - To evaluate cell viability, hOB1 cells were seeded at the density of 10000 cells/100µl/well in DMEM 10% FBS in 96 well-plates (Falcon, Becton Dickinson Europe, Meylan, France) containing the hydrogels in the presence or in the absence of anti-Fibronectin aptamers, after 1 hour pre-conditioning with HS-containing DMEM. Cell growth was assessed 10 days after seeding by MTT assay (Roche Applied Science, Penzberg, Germany) according to manufacturer's recommendations.

Cell encapsulation - For cell encapsulation, hOB1 cells were resuspended in DMEM enriched with 10% HS and mixed to thiolated Hyaluronic acid. PEGDA enriched with TE buffer or with Aptamers were added to the cell mixture and incubated for 1 hour to allow for the gels to set. After 10 days, gels were fixed with 4% Paraformaldehyde (Sigma-Aldrich), dehydrated through ethanol series, then embedded in Paraffin and sectioned. Sections were then stained with either Hematoxilin-Eosin or stained for fluorescence microscopy as described above.

Animals and housing - The study population consisted of female Wistar rats (Rattus Norvegicus, Charles River, Italy), 12-14 week of age, weighing 350-400 g. Animals were kept in unisexual groups of two individuals since weaning (4 week after birth) , supported until the start of the experiments in a temperature-controlled room at 22–24 °C, with lights on between 7.00 AM and 7.00 PM. The investigation was approved by the Veterinary Animal Care and Use Committee of the University of Parma.

In vivo scaffold implantation - Each animal received four subcutaneous pouches, one pouch for each gel/time point. At the time of implantation animals were anaesthetised with Zolazepam Tiletamine (30 mg/kg, i.p., Zoletil 100, Virbac, Italy). Two incisions were performed on the back of the animal in the interscapular region and subcutaneous pouches were then created to inoculate the hydrogels. The two pouches on the left side received 50µl each of hydrogel enriched with aptamers, the two pouches on the right side the control scaffold. The surgical wound was sutured with Prolene 4-0 (Johnson & Johnson, Amersfoort, The Netherlands) and animals were allowed to recover.

Histology - To evaluate the effects of aptamer-enriched hydrogels, animals were sacrificed at 4 and 7 days. Specimens were fixed in 4% formalin and, after 24 hours, embedded in paraffin. A section obtained from each animal heart was stained with Hematoxilin and Eosin. Microphotographs were taken with an optical microscope (Axioscope, Zeiss, Oberkochen, Germany) connected to a digital camera with final magnification of 100X.

Image analysis - Images were analysed with Image J (ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA).

Statistical analysis - Data were analysed using Prism 6 (GraphPad, La Jolla, CA, USA). All values are reported as the mean ± Standard Deviation of three repeated experiments. Differences between group means were evaluated with either t Test, one-way ANOVA, or two-way ANOVA statistical tests with Tukey post-test and differences were considered significant when $p<0.05$.

RESULTS

Aptamers enrich scaffolds for human Fibronectin

To investigate whether scaffolds functionalised with aptamers could be enriched for the target protein Fibronectin, hydrogels were prepared with aptamers at a concentration of 50 µg/ml and were incubated with concentrations of human Fibronectin comparable to those observed in culture conditions. Bradford assay showed a decrease in Fibronectin in surnatants when aptamers were present as compared to control hydrogel (Figure 2A, B) for both groups. Consistently with this, Bradford assay also revealed a higher amount of Fibronectin on aptamerenriched hydrogels as compared to control substrates (Figure 2C). The presence of Fibronectin was also investigated by immunofluorescence, using anti- Fibronectin antibodies (Figure 2D, E). Addition of aptamers greatly enhanced Fibronectin adsorption on hydrogels, as indicated by a greater degree of fluorescence. Fluorescence was also quantified and was shown to be significantly higher (Figure 2F).

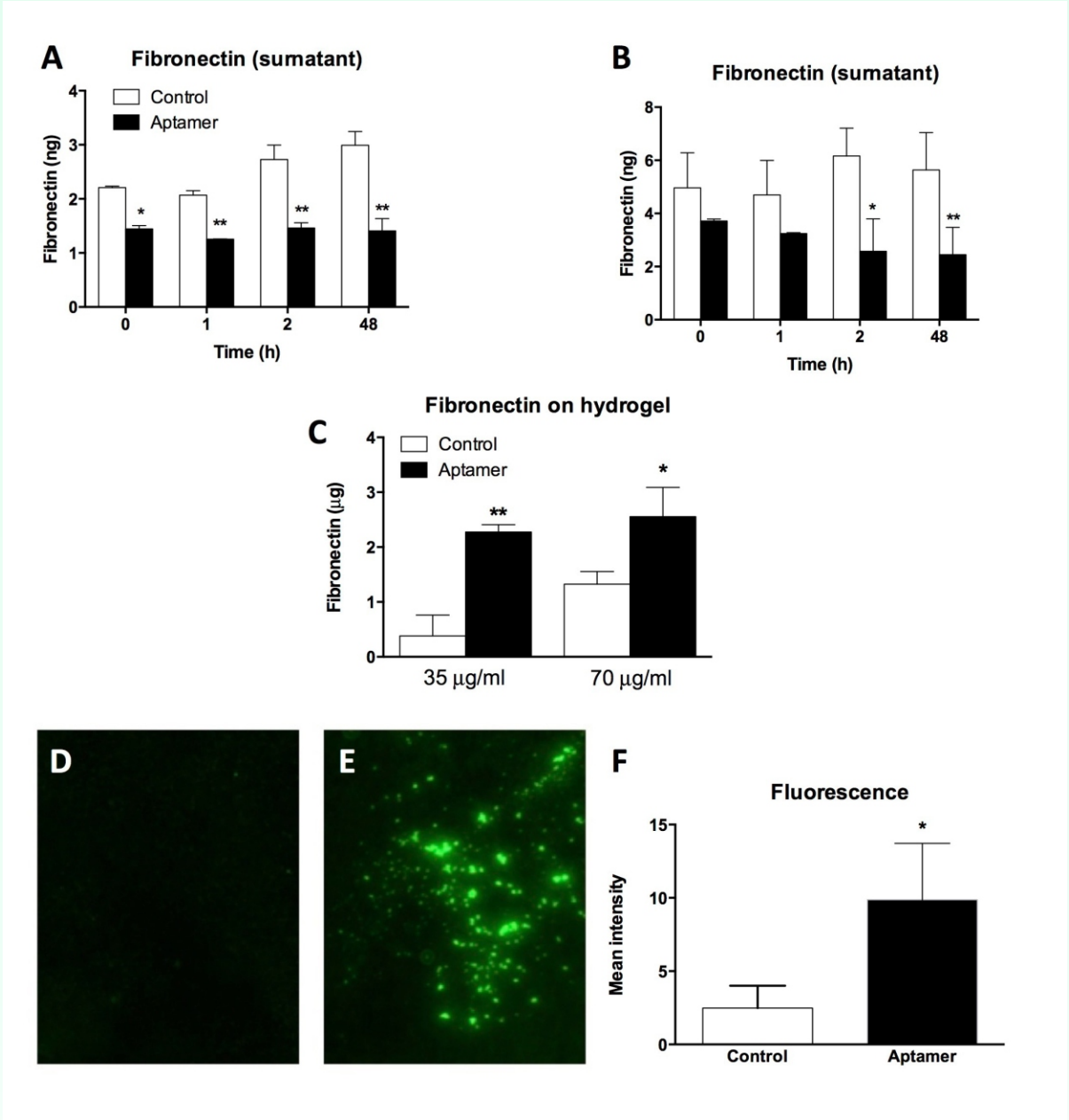


*Figure 2 Fibronectin binding on hydrogels. Fibronectin surnatant concentration starting from a 75 µg/ml (A) or 150 µg/ml (B) solution changes over time as these are incubated on funztionalized hydrogels and on controls gels, as measured by Bradford assay. (C) Amount of Fibronectin adsorbed on hydrogels by Bradford assay. (D-F) Functionalized hydrogels, after incubation with DMEM enriched with 10% human serum for 1 hour at 37°C and labelled with anti-Fibronectin antibody at immunofluorescence. (F) Fluorescence was quantitated using ImageJ software. Apatamers significantly enrich hydrogels selectivity for Fibronectin. *=P<0.05; **=p<0.001 versus control.*

Cell adhesion on hydrogel matrices is enhanced by anti- Fibronectin aptamers

Although few hOB cells were observed on control hydrogels after rinsing (Figure 3A, inset), suggesting poor cell attachment, many cells attached and proliferated on aptamerenriched gels, creating little cell clusters that covered the whole surface of the well (Figure 3A).
We quantitated cell number on Control and aptamer- enriched hydrogels by MTT assay after rinsing (Figure 3B). Cells on aptamer-containing hydrogels could be observed inside the gel, on multiple focus planes, whilst no such thing was encountered in control gels (data not shown). Cells cultured as described above were also fixed and stained for focal adhesions, microfilament and nuclei fluorescent labelling. More cells were observed on aptamer-enriched hydrogels and their cytoplasm appeared more spread and richer in adhesion complexes (Figure 3C, D).

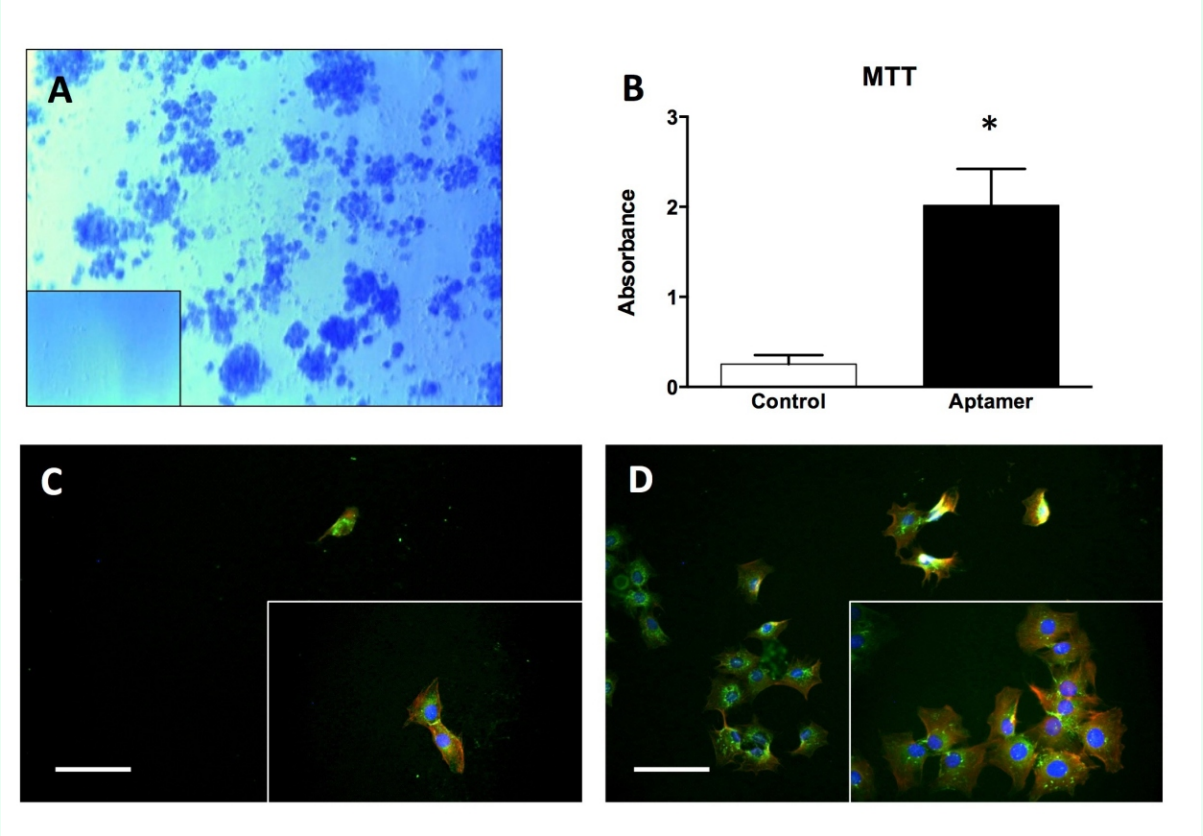


*Figure 3 (A) Hydrogels with or without aptamers (inset), after 10 days of cell culture in DMEM enriched with 10% Human Serum, after fixation and staining with Toluidine Blue, at inverted microscope. Magnification=100 X (B) Cell viability on control hydrogels or hydrogels containing aptamers, assessed at MTT assay. (C-D) Immunofluorescence observation of hOBs cultured on hydrogels in the absence or in the presence of aptamers. Cells were labelled for Vinculin (green), microfilaments (red) and nucleus (blue). Magnification 200 X, insets 400 X. Bar= 40 mm. *= p<0.05 versus control hydrogels.*

Cell colonisation of 3D matrices is enhanced by anti-Fibronectin aptamers

After 10 days, cells were present in both groups, as they had been encapsulated in the scaffold and thus more easily retained. However, beside cells being more numerous in the presence of aptamers (Figure 4A, B) average morphology appeared quite different: most cells were very elongated in the control group and possessed long, sometimes with neurite-like appearance, cytoplasmic extroflections. In contrast, cells in aptamer-containing gels had wider cytoplasm with broad podosomes, suggesting a firmer attachment to the substrate (Figure 4B). Fluorescence observation confirmed the presence of a higher number of cells in the presence of aptamers (Figure 4C, D).

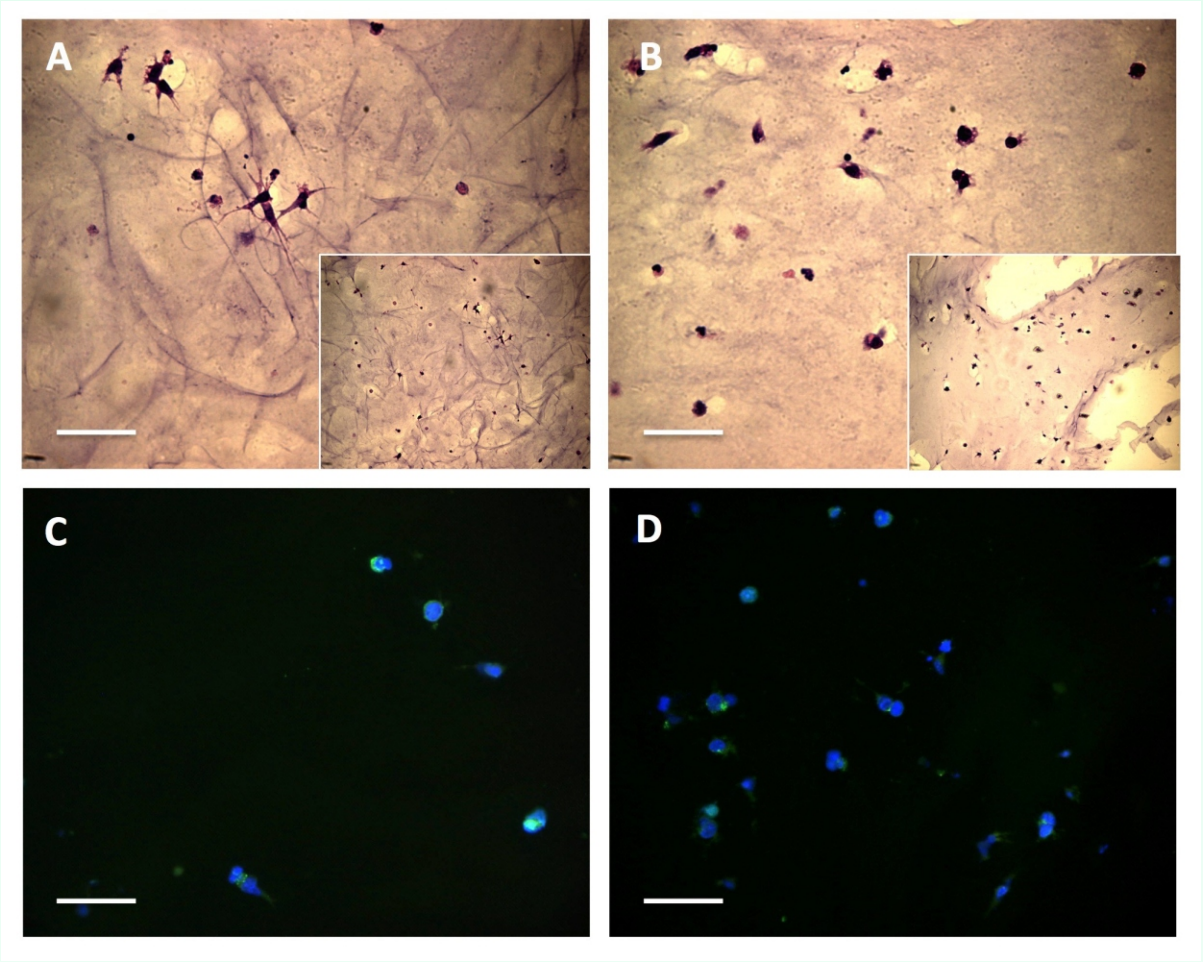


Figure 4 (A,B) Encapsulated hOBs in hydrogels in the absence (A) or in the presence (B) of anti- Fibronectin aptamers, after Hematoxilin-Eosin staining. Cells were more numerous in the presence of aptamers and their morphology was less elongated than in control hydrogels. (C,D) Encapsulated hOBs in hydrogels in the absence (C) or in the presence (D) of anti-Fibronectin aptamers, at immunofluorescence with nuclear labelling by DAPI and vinculin staining. Magnification 400 X, insets 200 X. Bar= 20 mm

Aptamers promote scaffold colonisation in vivo.

At day 4 day, it was possible to observe the presence of abundant granulation tissue around the implanted hydrogel in samples from both groups, as a product of the initial inflammatory process, though it seemed to be more abundant in control samples (Figure 5A,B). Cell infiltrate was still mostly haphazard, although an initial cell organisation could be noticed in aptamerenriched samples (Figure 5B). Signs of inflammation subsided in both groups by day 7,although, interestingly, hydrogels enriched with aptamers appeared to better promote scaffold invasion by cells, as indicated by the high density of fibroblastic cells, as compared with control samples at the same time point (Figure 5C,D). Cell infiltrate appears organised in areas of higher cell density, which are reminiscent of the cell clusters observed in vitro. Scaffolds from both groups appear populated by fibroblasts and small rounded lymphocyte-like cells. Taken together, these preliminary results suggest that anti-Fibronectin aptamers promote the colonisation of

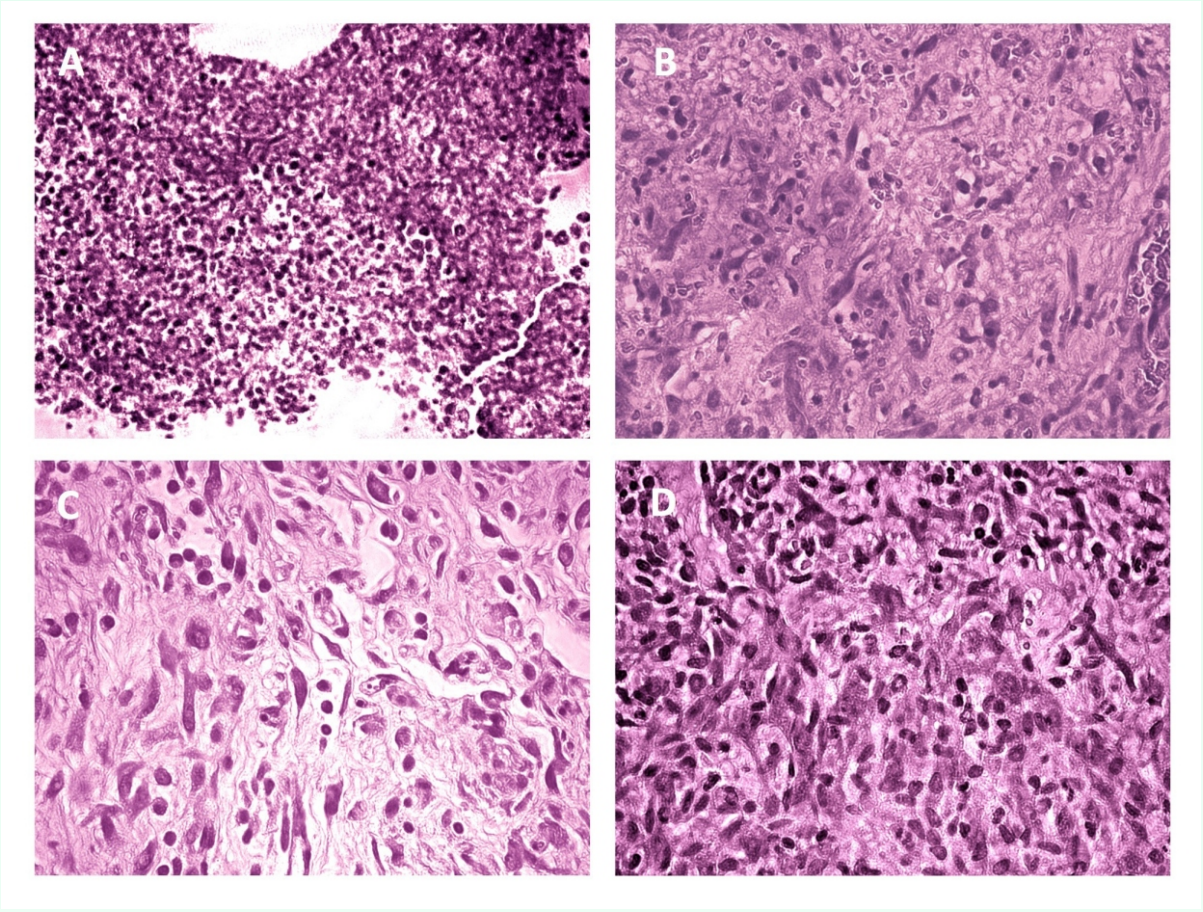


Figure 5 Histological sections of control (A, C) or aptamer-enriched (B, D) hydrogels implanted in rat dorsal sub-cute pouches and harvested 4 days (A, B) or 7 days (C, D) after surgery. Hematoxilin-Eosin staining, Magnification= 400 X. Higher cell density was observed in aptamer-enriched samples (D).

DISCUSSION

Biomaterials implanted into a tissue have a difficult goal to achieve, to integrate in such a complex environment and provide cells with a substrate which should as closely as possible mimic their native niche, to promote the attachment and scaffold colonisation by cell precursors, which will ultimately differentiate along the desired lineages and mediate tissue regeneration. Biomaterials can hardly exert any desired biological effect without adsorbing proteins on their surface. Although materials tend to spontaneously adsorb proteins when they are inserted into biological tissues, this process is mostly driven by protein availability and weak bonds established between protein species and material surface (Figure 1). A novel approach to selectively attract and retain desired proteins on biomaterial surfaces is to coat the implantable surface with elements able to dock specific target peptides. Aptamers can serve for such a purpose (Figure 1). Aptamers are oligonucleotides, whose primary and secondary structure enables them to recognise and bind specific target molecules (Tuerk and Gold, 1990), without eliciting adverse responses and being recognised by the host's immune system, because of their size and chemical nature (Keefe et al., 2010). Conventionally, aptamers are selected using the SELEX process (Systematic Evaluation of Ligands by Exponential Enrichment) (Ellington and Szostak, 1990), which ensures absence of variability in the end-product, in contrast to antibodies (Gold et al., 2010). Aptamers are also more stable to degradation and bind to their respective ligands under conditions not possible with antibodies (Jayasena, 1999).

In the present study PEGDA/tHA hydrogels were functionalised with anti-Fibronectin aptamers to enhance scaffold colonisation by cells. According to our results, anti-Fibronectin aptamers consistently promoted cell attachment and proliferation. We showed that aptamers could significantly bind and retain Fibronectin and this affected cell adhesion on the material. When cultured on the surface of the gels, few to no cell were visible in the control group after rinsing with PBS, whereas hOBs were observed on multiple focus planes in the presence of aptamers, which made focus difficult (Figure 3A). This suggests that aptamers promoted cell migration into the hydrogel. Noteworthy, aptamers promoted cell invasion of hydrogel scaffolds even in a rodent model of wound healing, although a lower binding affinity to rat Fibronectin was to be expected. A similar inflammatory process was observed at the earliest time point, which is not surprising as this is most likely driven by the bulk hydrogel material, which is the same in both experimental groups. However, after 1 week, a bigger and more organised cell infiltrate was visible in aptamer-enriched scaffolds. Further in vivo experiments will address longer time points, to investigate how aptamers affect tissue remodelling after wound healing and characterise the nature of the cell infiltrate to a greater extent.

In conclusion, our data support the idea that anti-Fibronectin aptamers improve cell adhesion to a hydrogel scaffold, such as could be used in a tissue defect. The choice of the target protein appears however to be critical, as novel properties can be conferred to medical devices by tuning the adsorbed protein species. Potentially, other bioactive molecules that are found in wounds could be targeted, such as PDGF, which is stored in platelet granules, to confer the scaffold with more specific biological effects, and that could be tuned according to the specific tissue to regenerate.

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