# A gene-activated injectable and 3D printable pro-chondrogenic hydrogel for the repair of articular cartilage defects

Donagh G. O'Shea[1,3], Tom Hodgkinson[1,2,3], James E. Dixon[4], Caroline M. Curtin[1,2,3], Fergal J. O'Brien[1,2,3]
[1] Tissue Engineering Research Group, Department of Anatomy and Regenerative Medicine, RCSI, Dublin, Ireland; [2] Trinity Centre for Biomedical Engineering, Trinity College Dublin, Ireland; [3] Advanced Materials and Bioengineering Research Centre (AMBER), RCSI and TCD, Dublin, Ireland; [4] Division of Regenerative Medicine & Cellular Therapies (RMCT), School of Pharmacy, University of Nottingham, UK.
**Email:** donaghoshea@rcsi.ie **Disclosures:** J.E.D. – (3C, 4, 7A), C.M. Curtin – (8), F.J. O'Brien – (3A, 3B, 5, 6, 7A, 8)

**INTRODUCTION:** Current treatments for articular cartilage (AC) repair are limited. However, hydrogels formulated using AC matrix components (such as hyaluronic acid; HA and collagen type II; Col II), offer a potential solution if they could be injected directly into the defect via minimally invasive arthroscopic procedures, or used as bioinks to 3D print customised regenerative scaffolds (Fig 1(a)). However, Col II and HA are notoriously difficult to incorporate into injectable and 3D printable (3DP) hydrogels due to poor physicochemical properties [1]. In this study, methacrylation of HA and the use of the FRESH method of 3D printing were investigated to overcome these issues [2]. Hydrogels can also be utilized for delivery of mesenchymal stem cells (MSCs), however it is challenging to stimulate and maintain their differentiation to articular-like chondrogenic phenotypes. Pro-chondrogenic factors such as TGF-β3 or SOX9 have been used to aid this; however the need for supraphysiological doses and issues with cellular delivery have hindered clinical translation [3]. Gene therapeutic nanoparticles (NPs), composed of plasmid DNA (pDNA) complexed to a non-viral delivery vector (e.g. the glycosaminoglycan enhanced transduction (GET) peptide [4]), offer a promising alternative by inducing cellular expression of therapeutic proteins, negating the need for high doses of exogenous proteins. However, clinical translation of this approach has proven challenging due to the short shelf-life of NPs, and so in this study, lyophilisation was investigated as a process to enable formulation of 'off-the-shelf' therapeutic NPs for incorporation in injectable/3DP hydrogels. In summary, this study aimed to develop a multi-faceted approach to cartilage repair involving development of a pro-chondrogenic HA and Col II injectable and 3DP hydrogel that was subsequently gene-activated with 'off-the-shelf' lyophilised NPs composed of the GET peptide complexed to pDNA for SOX9 (pSOX9) to direct enhanced cartilage repair.

**METHODS:** Methacrylated hyaluronic acid (MeHA) containing LAP photoinitiator was mixed in a 1:1 ratio with collagen type I (Col I), a Col I/Col II composite or Col II to form three hydrogels; MeHA-Col I, MeHA-Col I/Col II and MeHA-Col II. The rheological properties of the pre-gels were assessed via an amplitude and flow sweep under shear. The pre-gels were then photocrosslinked using blue light at 405nm and the swelling properties, as well as the compressive and dynamic moduli of the crosslinked hydrogels were assessed. Subsequently, degradation of the hydrogels was determined in a physiologically relevant enzyme solution [5]. The injectability of the pre-gels was assessed using a Zwick mechanical tester, while pre-gel 3D printing properties were determined using an Allevi 3 bioprinter with, and without, the use of mechanical support in the form of a gelatin slurry support bath (FRESH method [2]). Subsequently, MSCs ($5x10^6$ cells per mL) were incorporated into the hydrogels and cell viability in injected and 3D bioprinted scaffolds was assessed over 7 days. Following this, MSC-laden injected and 3D bioprinted scaffolds were cultured in TGF-β-containing (CCM) or TGF-β-free (ICM) media for 28 days and the deposition of AC matrix components was assessed using biochemical and histological assays with the aim of selecting an optimal hydrogel for injectable and 3D printing applications. Next, GET-pSOX9 NPs were lyophilised, stored at room temperature and the ability of the lyophilised NPs to up-regulate SOX9 protein production in 2D monolayer culture was determined. Finally, MSCs were transfected with lyophilised GET-pSOX9 NPs and incorporated into the optimal injected and 3D bioprinted hydrogel scaffolds and the deposition of AC matrix components was assessed following 28 days culture in CCM.

**RESULTS:** Each hydrogel possessed viscoelastic and shear thinning rheological properties and subsequent physicochemical analysis of the crosslinked hydrogels showed that Col II inclusion resulted in a more swollen and softer polymer network, without affecting degradation time. While all hydrogels exhibited exemplary injectability (Fig. 1(b)), only the Col I-containing hydrogels had the necessary mechanical stability for 3D printing applications. To facilitate 3D printing of multi-layered scaffolds using MeHA-Col I and MeHA-Col I/Col II, additional mechanical support in the form of a gelatin slurry support bath (FRESH method) was utilised (Fig 1(c)). Biological analysis revealed cells remained viable following injection and 3D printing and that Col II inclusion enhanced the deposition of AC matrix components such as sulfated glycosaminoglycans and Col II by MSCs, while suppressing deposition of the fibrocartilage component Col I in both injected and 3D printed scaffolds. Thus, MeHA-Col II was selected as the optimal injectable hydrogel, and MeHA-Col I/Col II as the preferred bioink. Subsequently, it was shown that lyophilised GET-pSOX9 NPs were as effective at up-regulating SOX9 protein production in MSCs as freshly complexed NPs. Finally, gene-activation of injected and 3D printed HA and Col II scaffolds with lyophilised GET-pSOX9 NPs resulted in significantly enhanced deposition of AC matrix components by MSCs, as evidenced by biochemical (Fig. 1(d)) and histological/immunohistochemical assays.

**DISCUSSION:** This study demonstrates the successful development of two HA and Col II implant types, through enhancement of hydrogel mechanical stability and shear thinning properties, which support cartilage regeneration. The MeHA-Col II hydrogel has suitable properties for use as an injectable hydrogel, while the MeHA-Col I/Col II hydrogel can used as an injectable hydrogel or as a bioink for 3D bioprinting. Overall, the addition of Col II results in a more swollen and softer polymer network, which is conducive to neocartilage formation. Lyophilisation was shown to be an effective method to enable formulation of 'off-the-shelf' gene therapeutics for regenerative medicine applications and gene-activation of the hydrogels with pro-chondrogenic 'off-the-shelf' NPs allowed for the fabrication of advanced biomimetic injected and 3D printed scaffolds with enhanced chondrogenic potential.

**CLINICAL RELEVANCE:** The versatility of the developed HA and Col II hydrogels allows for minimally invasive arthroscopic delivery, as well as 3D printing of scaffolds that match the clinical needs of the patient. Lyophilisation of the pro-chondrogenic NPs streamlines the gene-activated scaffold fabrication process, allowing clinicians or technicians to easily reconstitute the therapeutic and add it to the hydrogels at the point of use. Thus, the developed hydrogels help to overcome the shortfalls of current biomaterial scaffolds in the field and represent a promising development in the fabrication of patient-specific tissue implants to repair a notoriously difficult to treat tissue and, with refinement, this platform could also be used for the treatment of a myriad of other tissue types.

**REFERENCES: 1.** O'Shea (et al.), Biomater. Sci. 10:2462-2483, 2022, **2.** Hinton (*et al.),* Sci. Adv. 1(9):e1500758, 2015, **3.** Raftery (et al), Biomaterials 216:119277, 2019, **4.** Dixon (et al.), PNAS 113:3:E291-299, 2016, **5.** Moulisová (et al.), ACS Omega 2:11:7609-7620, 2017.
**ACKNOWLEDGEMENTS:** Funding: European Research Council under ERC grant agreement n°788753 (ReCaP).
**IMAGES AND TABLES:**

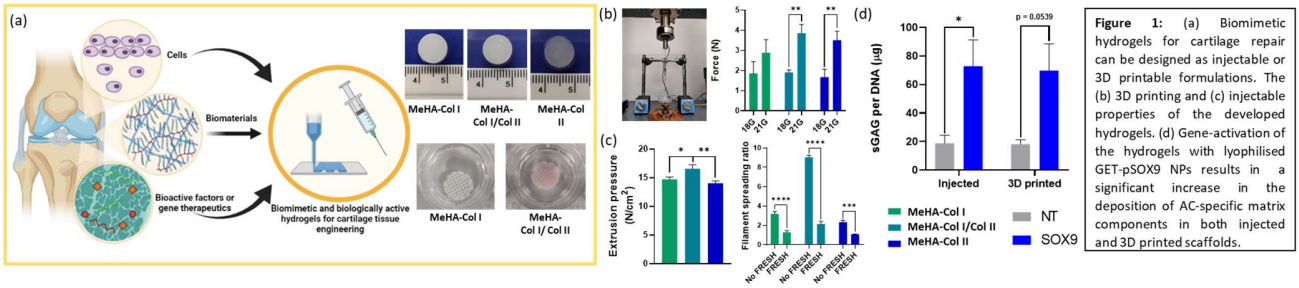


**Figure 1:** (a) Biomimetic hydrogels for cartilage repair can be designed as injectable or 3D printable formulations. The (b) 3D printing and (c) injectable properties of the developed hydrogels. (d) Gene-activation of the hydrogels with lyophilised GET-pSOX9 NPs results in a significant increase in the deposition of AC-specific matrix components in both injected and 3D printed scaffolds.