RESEARCH ARTICLE

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Peptoid-Cross-Linked Hydrogel Stiffness Modulates Human Mesenchymal Stromal Cell Immunoregulatory Potential in the Presence of Interferon-Gamma

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Human mesenchymal stromal cell (hMSC) manufacturing requires the production of large numbers of therapeutically potent cells. Licensing with soluble cytokines improves hMSC therapeutic potency by enhancing secretion of immunoactive factors but typically decreases proliferative ability. Soft hydrogels, however, have shown promise for boosting immunomodulatory potential, which may compensate for decreased proliferation. Here, hydrogels are cross-linked with peptoids of different secondary structures to generate substrates of various bulk stiffnesses but fixed network connectivity. Secretions of interleukin 6, monocyte chemoattractive protein-1, macrophage colony-stimulating factor, and vascular endothelial growth factor are shown to depend on hydrogel stiffness in the presence of interferon gamma (IFN-) supplementation, with soft substrates further improving secretion. The immunological function of these secreted cytokines is then investigated via coculture of hMSCs seeded on hydrogels with primary peripheral blood mononuclear cells (PBMCs) in the presence and absence of IFN- . Cocultures with hMSCs seeded on softer hydrogels show decreased PBMC proliferation with IFN- . To probe possible signaling pathways, immunofluorescent studies probe the nuclear factor kappa B pathway and demonstrate that IFN- supplementation and softer hydrogel mechanics lead to higher activation of this pathway. Overall, these studies may allow for production of more efficacious therapeutic hMSCs in the presence of IFN- .

1. Introduction

Human mesenchymal stromal cells (hMSCs) have emerged as a promising source for cell-based therapeutic applications.[1–3] Their clinical potential has been assessed with regard to a wide variety of disorders and diseases, including cancer,[4] diabetes,[5] multiple sclerosis,[6] cardiovascular disease,[7] neurodegenerative disorders,[8] autoimmune disorders,[9] and inflammatory diseases.[10] Some advantages of hMSCs for applications in human health are their lack of significant ethical concerns, their ease of harvesting, and their relatively low risk of tumorigenesis.[2,11–13] In addition, hMSCs have garnered interest due to their secretome, which is the collection of secreted molecules produced by the cells.[14–17] The hMSC secretome is a complex mixture of various cytokines, growth factors, and extracellular matrix components that play a crucial role in cellular communication and regulation of the surrounding microenvironment.[18] The secretome has been shown to have anti-inflammatory and immunomodulatory effects, which has led to further interest in strategies to control its composition for therapeutic applications, including for autoimmune and degenerative conditions.[19,20] That said, there are still very few hMSC therapies approved for the clinic, due in part to the dosing requirements needed for therapeutic benefit. This has prompted significant interest in the production of more therapeutically relevant hMSCs with controllable secretomes and higher levels of immunoregulatory and regenerative cytokines.

One area of emerging interest for optimizing the therapeutic efficacy of hMSCs is to leverage the interplay of the culture scaffold and traditional preactivation processes. Traditionally, hMSCs are preactivated, or "licensed," with proinflammatory cytokines such as interferon-γ, resulting in increased secretion of immunoactive factors such as indoleamine 2,3-dioxygenase (IDO).[21–26] Numerous cell culture scaffolds have been designed to replicate the physical, chemical, and mechanical characteristics of the native extracellular matrix to produce more therapeutically relevant hMSCs.[27–29] Current scaffolding technologies include sponges,[30,31] nanofibers,[32–34] multilayers,[17,35,36] and hydrogels,[29,37] each of which has been explored with various molecular compositions.[38] While these studies have offered some insight to the factors that control the hMSC secretome, biophysical cues, especially the matrix stiffness, are less well studied

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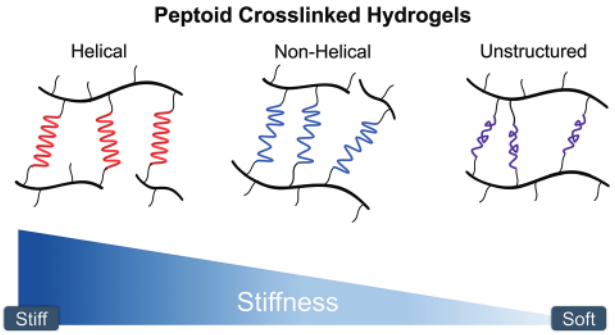
as a preconditioning strategy, despite their broad applicability to numerous scaffolding types.[39]

Matrix stiffness is well-known to influence hMSC phenotype, and hydrogel platforms in particular provide an easily tunable substrate for investigating stiffness effects on the hMSC secretome. In general, a variety of hydrogel scaffolds have been found to enhance the immunomodulatory potential of licensed hMSCs, compared to cells cultured on plastic.[16,39–48] However, there is debate as to the ideal stiffness range to promote beneficial immunomodulatory effects.[43] Abdeen et al. found that hMSCs cultured on 2D polyacrylamide hydrogels showed increased vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 production on stiffer hydrogels ($E \approx 40$ kPa) than on softer counterparts ($E \approx 500$ Pa),[41] while Ogle et al. showed that hMSCs cultured on 30 kPa polyethylene glycol (PEG) hydrogels showed significantly more secretion of many immunomodulatory factors than on stiffer 100 kPa hydrogels.[42] Wong et al. stimulated hMSCs in soft ($E \approx 2$ kPa) and stiff ($E \approx 35$ kPa) hydrogels with tumor necrosis factor alpha (TNF-), leading to increased monocyte production with cells cultured in soft substrates,[43] and Zhuang et al. showed that hMSCs in methacrylated gelatin hydrogels of similar "soft" conditions induced more macrophages to an anti-inflammatory phenotype. Thus, matrix stiffness clearly impacts the hMSC secretome, but a complete understanding is not yet developed.[46]

One possible reason for the variation in these results is that it is difficult to control hydrogel stiffness without also altering some combination of permeability, swelling ratio, or adhesive ligand density. Matrix stiffness is usually controlled by altering polymer concentration or cross-link density in the hydrogel, which is intrinsically coupled to the mesh size. To decouple these variables and isolate the effects of matrix stiffness, we previously developed a hydrogel platform that leverages the chain stiffness of the cross-linkers to control bulk elasticity.[40,49] In both PEG and hyaluronic acid hydrogels, we showed that matrix stiffness increased when we used stiff, helical peptoid cross-linkers compared to more flexible, disordered peptoid cross-linkers, while fixing the network connectivity to hold the permeability and swelling ratio constant. Initial studies of hMSCs cultured on these substrates showed marked differences with stiffness in cellular spread area, circularity, and proliferation, with softer hydrogels ($E \approx 1.5$ kPa) leading to increased secretion of IDO both in the presence and absence of licensing with interferon gamma (IFN-γ).[40] These results indicated the potential to enhance the therapeutic efficacy of licensed hMSCs using tunable hydrogels of soft-to-intermediate stiffness.

Whereas our previous work demonstrated proof of concept with IDO secretion, the work here investigates the secretory activity and function of hMSCs using our previously developed peptoid-cross-linked hydrogel platform. To gain a deeper understanding of how substrate stiffness controls hMSC immunomodulatory potential, here we investigated the secretion of seven additional human cytokines and chemokines from hMSCs seeded on our hydrogels, including interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), macrophage colony-stimulating factor (M-CSF), VEGF, epidermal growth factor (EGF), interferon-gamma-induced protein 10 (IP-10), and fibroblast growth factor 2 (FGF-2), which have been shown to play key roles in hMSC immunoregulation.[50] We also evaluated the immunosuppressive properties of hMSCs cultured on peptoid-cross-linked hydrogels by assessing suppression of peripheral blood mononuclear cell (PBMC) proliferation during direct contact coculture experiments. PBMCs are a mixture of multiple immune cell types, including T-cells, B-cells, natural killer cells, monocytes, and dendritic cells, and are the primary cells associated with human immune response.[51] Because of their relevance to human body immunity, PBMCs have been used as a model immune system.[52] To probe intracellular signaling pathways linking matrix mechanics to secretion, we studied the activation of the nuclear factor kappa B (NF-κB) signaling pathway for hMSCs seeded on the developed hydrogels. Finally, we further assess ability of the hMSCs to differentiate into multiple lineages, namely adipogenic and osteogenic, after culture on our developed substrates. This study uniquely probes hMSC immunomodulatory potential on substrates where mechanics are decoupled from network connectivity. These results have important implications for the development of hydrogels for use in hMSC manufacturing, where the mechanical properties of the hydrogels will impact the behavior of cells on the hydrogel, their secretome, and ultimately, the therapeutic outcome.



Scheme 1. Peptoid cross-linked hydrogels using sequence-defined secondary structure: helical (Hel), nonhelical (N-hel), and unstructured (Unst).

2. Results and Discussion

2.1. Cross-Linker Structure Determines Hydrogel Properties

Three different peptoid cross-linkers were synthesized, each of which had different chain stiffnesses according to its sequence-defined secondary structure: helical (Hel), nonhelical (N-hel), and unstructured (Unst) (**Scheme 1**). The Hel and N-hel peptoids contain chiral sidechains, which increase their chain stiffness compared to the Unst peptoid, which is completely achiral. Additionally, a peptide cross-linker (KCGGIQQWGPCK) (Figure S4, Supporting Information) was synthesized as an additional control because it contains a chiral backbone, but no designed secondary structure, and has been shown to yield a modulus intermediate to the N-hel and Unst peptoid hydrogels.[40] All hydrogels were prepared using photoinitiated thiol–ene cross-linking with a norbornene-functionalized hyaluronic acid, as described previously.[40] We have shown that the hydrogels resulting from these different cross-linkers span a swollen elastic moduli range from ≈1.5 to 12 kPa.[40]

Fourier-transform infrared spectroscopy (FTIR) was used to assess the chemical composition of the cross-linked hydrogels.

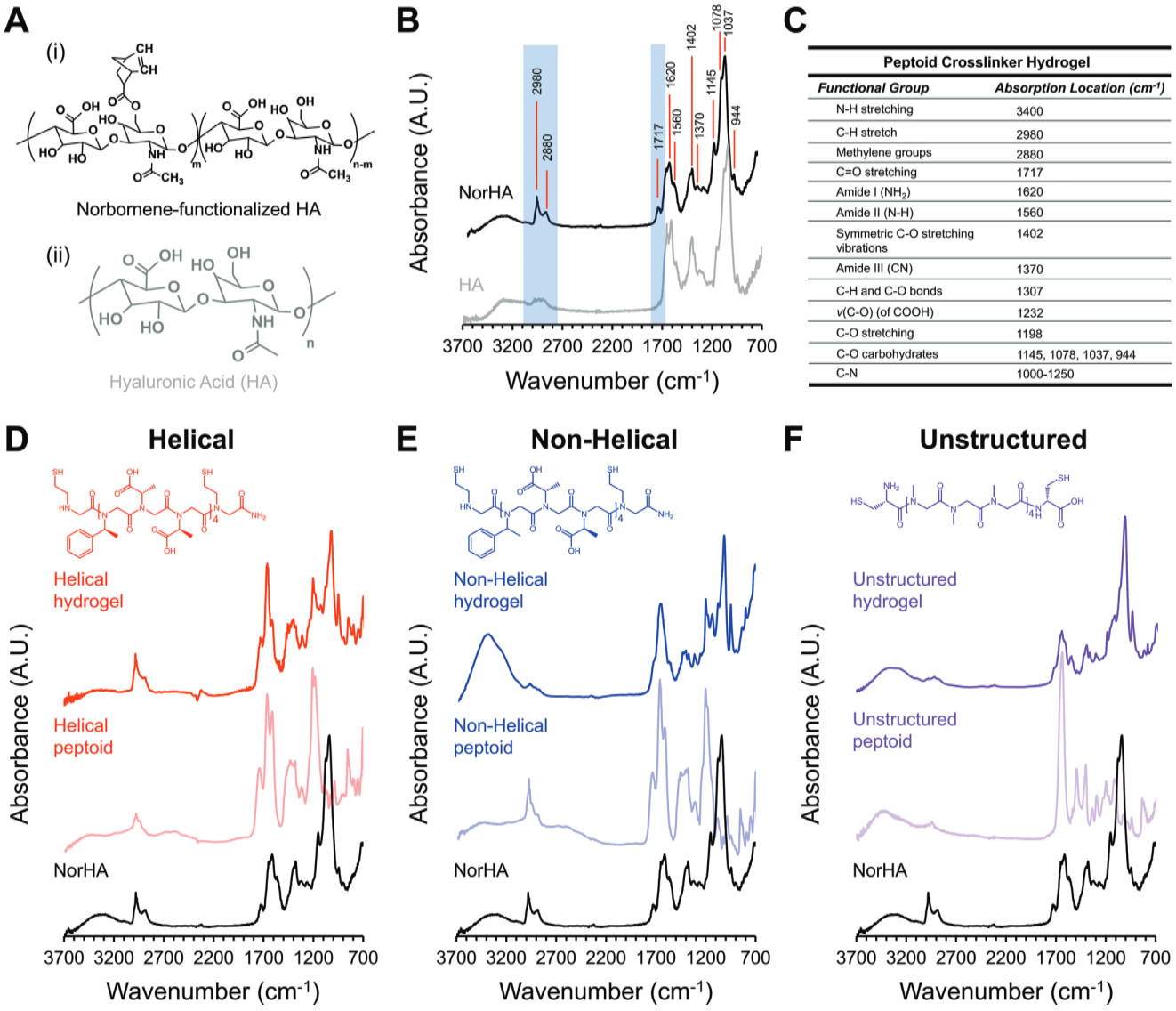


Figure 1. FTIR analysis confirming the inclusion of the cross-linker and the maintenance of the hyaluronic acid peaks for each formulation. Chemical composition of hyaluronic acid, norbornene-functionalized hyaluronic acid, and peptoid-cross-linked hydrogels. A) Chemical structure i) HA, ii) norbornene-functionalized HA, B) FTIR spectrums of HA and norbornene-functionalized HA (range: 3700–700 cm^{-1}). C) Functional groups versus absorption location. FTIR spectrums of the peptoids and peptoid-cross-linked hydrogels (range: 3700–700 cm^{-1}). D) Helical, E) nonhelical, F) unstructured.

Hydrogels were prepared in a mixture of dimethyl sulfoxide (DMSO) and phosphate-buffered saline (PBS), then washed to remove DMSO and freeze-dried. The dried hydrogels were analyzed with FTIR (**Figure** 1C–F). Because hyaluronic acid (HA) is a linear polysaccharide, it is expected that the FTIR spectrum would show characteristic absorption peaks at 1620, 1560, and 1370 cm^{-1}, corresponding to NH_2 (amide I), N—H bending (amide II), and C—N stretching (amide III), respectively (Figure 1A–C).[53,54] Other notable features for HA include broad peaks around 1402 and 1037 cm^{-1} for C—O stretching, as well as O—H and N—H stretching vibrations around ≈3400 cm^{-1} (Figure 1B). After functionalization with norbornene, characteristic adsorption peaks associated with the C—H stretch (2980 cm^{-1}), methylene groups (2880 cm^{-1}), and C═O stretching (1717 cm^{-1}), were detected (Figure 1A–C).[53,55]

For all peptoids, a strong contribution of amide I around 1665 cm^{-1} was observed (Figure 1C–F). As expected, helical and nonhelical peptoids presented similar chemical composition since they only differ in chirality (Figure 1D–F). The predominate peaks for the helical and nonhelical peptoids can be attributed to ring C—H stretching vibrations (2900–3000 cm^{-1}), C═O stretching groups (1717 cm^{-1}), amide I (1665 cm^{-1}), and N—H (1470 cm^{-1}) (Figure 1D–F). The unstructured peptoid displayed a unique spectrum with characteristic peaks associated with the amide I (1665 cm^{-1}), N—H (1490 cm^{-1}), and C—N (1000–1250 cm^{-1}) stretching (Figure 1D–F). Evaluating the FTIR spectra