Imparting Immunomodulatory Activity to Scaffolds via Biotin–Avidin Interactions

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ABSTRACT: Biotin–avidin interactions have been explored for decades as a technique to functionalize biomaterials, as well as for in vivo targeting, but whether changes in these interactions can be leveraged for immunomodulation remain unknown. The goal of this study was to investigate how biotin density and avidin variant can be used to deliver the immunomodulatory cytokine, interleukin 4 (IL4), from a porous gelatin scaffold, Gelfoam, to primary human macrophages in vitro. Here, we demonstrate that the degree of scaffold biotinylation controlled the binding of two different avidin variants, streptavidin and CaptAvidin. Biotinylated scaffolds were also loaded with streptavidin and biotinylated IL4 under flow, suggesting a potential use for targeting this biomaterial in vivo. While biotin–avidin interactions did not appear to influence the protein release in this system, increasing degrees of biotinylation did lead to increased M2-like polarization of primary human macrophages over time in vitro, highlighting the capability to leverage biotin–avidin interactions to modulate the macrophage phenotype. These results demonstrate a versatile and modular strategy to impart immunomodulatory activity to biomaterials.

KEYWORDS: biotin–avidin, drug delivery, macrophage polarization, immunomodulation, interleukin 4

INTRODUCTION

Implantation of biomaterials triggers the inflammatory response due to the creation of an injury and the presence of a foreign material in the body. This critical response protects the body from pathogens and promotes tissue repair and regeneration. However, in the presence of biomaterials, this response can transition from healing the tissue to the foreign body response (FBR) (for review, see ref 1). The FBR encapsulates the biomaterial in a dense, fibrous matrix, isolating it from surrounding tissues and impeding regeneration.

Macrophages, a major cell type of the innate immune response, are one of the critical regulators of the FBR. Macrophages orchestrate eventual scaffold resorption and coordinate the formation and remodeling of new tissue, depending on the biomaterial’s biophysical and biochemical microenvironments. In order to actively induce the time-dependent transitions of macrophage phenotype to overcome the FBR, or to stimulate functional tissue regeneration for in situ tissue engineering applications, new drug delivery platform technologies are required to modulate the macrophage phenotype in a temporally controlled way.

Biotin has extremely high specificity and affinity for the protein avidin ($K_d = 10^{-15}$ M) and its variants, such as streptavidin ($K_d = 10^{-14}-10^{-15}$ M). Both avidin and streptavidin have four binding sites for biotin, meaning that they can bind up to 4 mols of biotin per mol of protein. CaptAvidin is a nitrated version of avidin that is designed to

This functional pleiotropy of macrophages has inspired the development of biomaterial-driven, in situ tissue regeneration strategies, which employ resorbable scaffolds to induce endogenous tissue regeneration via immunomodulation (for reviews, see refs 5 and 6). Macrophages orchestrate eventual scaffold resorption and coordinate the formation and remodeling of new tissue, depending on the biomaterial’s biophysical and biochemical microenvironments. In order to actively induce the time-dependent transitions of macrophage phenotype to overcome the FBR, or to stimulate functional tissue regeneration for in situ tissue engineering applications, new drug delivery platform technologies are required to modulate the macrophage phenotype in a temporally controlled way.

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associate with biotin at pH greater than 4 and completely dissociate at a pH greater than 10 ($K_d = 10^{-7}$ M at pH 4). This reversible interaction is useful in biosensor applications. However, due to further postproduction modifications, CaptAvidin can only bind up to 2.7 mols of biotin per mol of protein. This decreased binding capacity of CaptAvidin for biotin leads to more transient association with biotinylated proteins. Due to these strong interactions, avidin and its variants have been widely used as bioconjugation systems to attach proteins, and even cells, to biotinylated biomaterials. Moreover, biotinylated drugs have been delivered to avidin-bound targets in vivo via systemic administration, further demonstrating the utility of this platform. Finally, the release of biotin conjugates has even been controlled by leveraging the faculty that biotin conjugates have lower affinity for avidin compared to free biotin.

The goal of this study was to determine how biotin density and avidin variant affect the loading of biotinylated IL4 and the resulting temporal effects on primary human macrophages in vitro (Figure 1). Using a model biomaterial of Gelfoam, a porous gelatin scaffold, we created various modified scaffolds by modulating biotin—avidin conjugation parameters (Figure 1A). To explore the potential for in situ loading of materials directly exposed to high blood flows, such as cardiovascular implants of heart valves or blood vessels, we studied how this system might be used to target a biomaterial under flow conditions (Figure 1B). Finally, we investigated the effects of the degree of biotin conjugated to the scaffolds and avidin variants, CaptAvidin and streptavidin, on the polarization behavior of primary human macrophages over time in vitro (Figure 1C).

**Figure 1.** Biomaterial and experimental design. (A) Process steps for creating a biotin—avidin-mediated drug delivery system. (B,C) Studies conducted to investigate the multiple aspects of the biomaterial system. FME, fold molar excess.

### MATERIALS AND METHODS

**Scaffold Preparation.** Porous gelatin scaffolds (Gelfoam, Pfizer Inc.) were biopsy punched (diameter = 5 mm) and cross-linked with either 0 or 0.1% glutaraldehyde in 1× phosphate-buffered saline (PBS) at either room temperature for static experiments or 4 °C for dynamic experiments for 24 h. Scaffolds were then washed in 1× PBS at least five times, 5 min per wash, to remove any unreacted glutaraldehyde. Scaffolds were stored at 4 °C until further use.

To sterilize scaffolds before further modification for bioactivity studies, scaffolds were soaked in 70% ethanol for 45 min, then washed in sterile 1× PBS at least five times, 5 min per wash, to remove any residual ethanol. Sterilized scaffolds were stored at 4 °C until further use.

**Biotinylation of Scaffolds.** Scaffolds were incubated for 1 h at room temperature with 0.1-, 1-, 10-, or 100-fold molar excess (FME) of sulfo—NHS—LC—biotin (Pierce Premium Grade, Thermo Fisher) for the covalent conjugation to available primary amines on the scaffolds. FME of sulfo—NHS—LC—biotin was calculated based on the molecular weight of gelatin and the mass of the scaffold. After 1 h, biotinylated scaffolds were washed at least five times, 5 min per wash, in PBS to remove any unbound sulfo—NHS—LC—biotin. For bioactivity studies, these steps were carried out under sterile conditions and using sterile biotinylation reagents and sterile PBS.

To measure the degree of biotinylation as a function of FME sulfo—NHS—LC—biotin added, the HABA (4′-hydroxyazobenzene-2-carboxylic acid)—avidin assay was conducted following previously established methods.

**Binding Biotinylated Scaffolds with Avidin Variants.** Biotinylated scaffolds were incubated with streptavidin (Thermo Fisher Scientific) or CaptAvidin (Thermo Fisher Scientific), avidin variants with different affinities for biotin, and avidin variants were incubated for 1 min at room temperature. After reacting for 1 min, the absorbance of the supernatant was read at 500 nm.

To confirm binding of avidin variants to biotinylated scaffolds, fluorescent avidins were used. For CaptAvidin-modified scaffolds, CaptAvidin was first conjugated with primary amine reactive,
fluorescent DyLight 350 NHS Ester (Thermo Fisher) by incubating 3.5 μM CaptAvidin with 10 FME of DyLight overnight at 4 °C. The following day, the CaptAvidin–DyLight solution was dialyzed (7000 Da molecular-weight cutoff dialysis cassette, Thermo Fisher) for 4 h at room temperature in 500 times excess PBS, with a media change every hour, to remove any unconjugated DyLight. Following dialysis, CaptAvidin–DyLight was incubated with both biotinylated and unbiotinylated scaffolds for 1 h at room temperature, followed by five washes, 5 min per wash, with 1X PBS. For streptavidin-modified scaffolds, 10 μM fluorescent streptavidin (Fstrep) (streptavidin, Alexa Fluor 488 conjugate, Thermo Fisher Scientific) was used following preliminary studies where 2.5 μM streptavidin was below the limit of detection. Fstrep was incubated with both biotinylated and unbiotinylated scaffolds for 1 h at room temperature, followed by washing at least five times, 5 min per wash, in PBS.

After the final wash step, fluorescently labeled scaffolds were imaged using a Leica DMi6000B inverted microscope. The mean fluorescence intensity of each scaffold was quantified using ImageJ to measure the amount of bound avidin variant on biotinylated scaffolds compared to non-biotinylated controls.

Fstrep was also used to confirm complete removal of residual glutaraldehyde after cross-linking. These scaffolds were quenched with glycine after cross-linking with glutaraldehyde, biotinylated, associated with Fstrep, and imaged, as described above.

**Loading Fluorescent Streptavidin under Flow Conditions.**

To determine whether the degree of biotinylation affects binding of Fstrep to biotinylated scaffolds under flow conditions, which would occur for intravascular biomaterials, biotinylated scaffolds were loaded into a previously developed parallel plate flow chamber (channel height = 200 μm, width = 5 mm).²³ Scaffolds were exposed to fluorescently labeled streptavidin at 4 μg/mL under laminar flow conditions (14.3 mL/min), imposing physiological shear stress levels of 5 Pa onto the surface of the scaffold (Supporting Information, S1).

First, the scaffold was exposed to 1X PBS (37 °C) to set the baseline intensity and allow the flow to stabilize. At t = 10 min, the streptavidin Alexa Fluor 488 conjugate (Fstrep, Molecular Probes), diluted in PBS, was added to the system via an in-line injection port (Ibidi GmbH), bringing the final dilution in the flow system to 1:500 (v/v) or 0.67 nmol in 10 mL. At t = 40 min, the circulating solution was replaced with PBS without any Fstrep to remove potential background intensity from unbound Fstrep.

To measure Fstrep binding to the scaffold, images of the scaffold under flow were taken with a fluorescent light microscope (Leica DMi8 TIRF Primo, Leica microsystems CMS GmbH, Mannheim, Germany) every 30 s over a period of 50 min at 37 °C. Mean intensity of the images was quantified over time using OME Bioformats plugin for ImageJ software.²⁴²⁵ The final increase in intensity was calculated by subtracting the baseline intensity from the mean intensity after the change in PBS (t > 40 min).

**Biotinylation of IL4.**

Human recombinant interleukin 4 (IL4) (PeproTech, 5 μg/mL) was conjugated with 10 FME of biotin via incubation with sulfo-NHS–LC–biotin (Pierce Premium Grade, Thermo Fisher) for 1 h at room temperature. The biotinylated IL4 (bIL4) solution was then dialyzed (3000–7000 Da molecular-weight cutoff, Thermo Fisher) in 500 times excess PBS overnight to remove any unconjugated biotin from the solution, with two media changes after 2 and 4 h. After dialysis, if scaffolds were being used for static or flow bioactivity studies, the bIL4 was sterile filtered using a sterile syringe filter (0.2 μm, Millipore Sigma) before incubation with the scaffolds. Otherwise, bIL4 was immediately incubated with modified scaffolds. For the static studies, 1 μg bIL4 in 0.2 mL was used. For the flow studies, 5 μg bIL4 in 0.5 mL was injected in the system via an in-line injection port following the establishment of stable flow for 10 min. After 1 h, the scaffolds were removed from the flow system.

Because the HABA–avidin assay is not sensitive enough to detect the relatively small quantities of biotin that were bound to IL4, conjugation of a fluorescent molecule of a similar size and primary amine binding mechanism was quantified to approximate how changes in FME would affect the mols of sulfo-NHS–LC–biotin binding per IL4 molecule. IL4 was incubated with 1-, 10-, or 100-μM NHS-DyLight 350 (Thermo Fisher) in 1X PBS for 8 h at 4 °C. Then, to remove any unreacted DyLight 350, samples were dialyzed (7000 Da molecular-weight cutoff dialysis cassette, Thermo Fisher) in 500 times excess PBS, with media changes every hour for 4 h, before dialyzing overnight. A standard curve of known quantities of DyLight 350 ranging from 0 to 5 μg/μL was used to interpolate the amount of DyLight 350 bound to IL4.

**Quantifying Release of IL4 from Modified Scaffolds.**

Biotinylated IL4 (1 μg) was added to each modified scaffold for 1 h at room temperature. Scaffolds were then washed three times, 5 min per wash, with PBS. After the last wash, modified scaffolds were immediately used for studies. To account for nonspecific adsorption of bIL4 to scaffolds, the adsorbed IL4 control group was prepared with 1-FME of sulfo-NHS–LC–biotin and bIL4 but no avidin variants. To determine the potential of protein adsorption-mediated binding and release, two versions of this control group were prepared: one that was washed three times, 5 min per wash, to exactly match the avidin-containing experimental groups (Ads IL4), and one that did not undergo the wash step at all (unwashed). All scaffolds were incubated in either 1X PBS or 10 mM biotin solution (excess biotin condition) at 37 °C and 75 rpm for 10 days. Supernatant was collected at 1, 6, 12, and 24 h, and then every 3 days thereafter (n = 5 scaffolds per group). Supernatant was completely exchanged at each time point. Enzyme-linked immunosorbent assay (ELISA) (PeproTech) was used to quantify the amount of IL4 in the supernatant at each time.

**Validation of Bioactivity of IL4 Following the Biotinylation Procedure.**

To verify the bioactivity of bIL4 in terms of stimulating M2 polarization, macrophages seeded in six-well plates were cultured for 2 days with complete media, supplemented with either 40 ng/mL bIL4 or 40 ng/mL unbiotinylated IL4. M1 and unpolarized (M0) macrophages were included as controls.²⁶ After 2 days, cells were either directly used for flow cytometry measurements to determine surface marker expression (Supporting Information, S2) or lysed (RTL-lysis buffer, Qiagen, Venlo, Netherlands) and stored at −80 °C until the quantitative reverse transcription polymerase chain reaction (qRT-PCR). The experiment was repeated for three different donors for flow cytometry and two different donors for qRT-PCR.

**Bioactivity of Modified Scaffolds on Macrophage Polarization In Vitro under Static Conditions.**

Peripheral blood-derived monocytes, purchased from the University of Pennsylvania Human Immunology Core, were differentiated into M0 macrophages following a previously established protocol for 5 days, with a media change at day 3.²³ Prior to seeding macrophages on 0.1% glutaraldehyde cross-linked, biotinylated scaffolds, via streptavidin or CaptAvidin and bIL4, scaffolds were incubated in basal media for 30 min, which was entirely removed before cells were added. Macrophages were removed from tissue-culture-treated polystyrene flasks by gentle scraping and seeded at 5 × 10⁵ cells per scaffold. The cell suspension was directly pipetted onto the scaffolds in 10 μL and incubated for 30–45 min to promote the cell attachment. Complete media (RPMI 1640 + 10% heat-inactivated human serum + 1% penicillin streptomycin) was then added to fully submerge the scaffolds. Media was collected every 2 days and entirely replaced with fresh complete media. On day 14, conditioned media was collected, and scaffolds were placed in Trizol, then stored at −80 °C until further processing.

**Preparation of Modified Scaffolds under Flow Conditions.**

Biotinylated scaffolds were prepared, as described in the previous sections. Then, for the flow group, a scaffold was placed in the flow chamber and connected to the flow loop in the incubator (37 °C, 5% CO₂). Syringes were filled with 4.5 mL of PBS (37 °C) and exposed to a flow of 14.3 mL/min (r = 5 Pa, Supporting Information, S1). After approximately 10 min, a stable flow was established, and 5 μg of bIL4 in 0.5 mL was injected into the system via an in-line injection port. After 1 h, the scaffold was removed from the flow system. For the statically loaded groups (Ads IL4 and static), 1 μg of bIL4 in 1 mL of PBS was added to the scaffolds and incubated for 1 h in the incubator (37 °C, 5% CO₂). The final concentration of IL4 during the loading phase was 1 μg/mL for both the static and flow groups. After...
incubation, scaffolds are washed five times with sterile PBS to remove unbound IL4.

**Monocyte Isolation and Differentiation for the Biotinylated IL4 Bioactivity Studies and Flow Conditions.** For experiments conducted under flow, human peripheral blood buffy coats were obtained from healthy, anonymized volunteers under informed consent, approved by the Sanquin Research Institutional Medical Ethical Committee (Sanquin, Nijmegen, the Netherlands). Buffy coats were diluted in 0.6% (w/v) sodium citrate (sodium citrate dehydrate trisacid, Sigma-Aldrich) in PBS. Peripheral blood mononuclear cells (hPBMCs) were isolated using density gradient centrifugation on iso-osmotic medium with a density of 1.077 g/mL (Lymphoprep, Axis Shield, Oslo, Norway). hPBMCs were repetitively washed with 0.6% ice-cold sodium citrate, resuspended in cell culture medium (RPMI + 2% heat inactivated human serum + 1% penicillin streptomycin), and seeded with a concentration of 1.3 × 10^6 cells/cm² in either T75 flasks or in six-well plates (Greiner). After cell-seeded scaffolds were diluted in 0.6% (w/v) sodium citrate (sodium citrate dehydrate trisacid, Sigma-Aldrich) for 1 h at 4 °C, followed by gentle scraping. Prior to cell seeding, 0.1% glutaraldehyde cross-linked, biotinylated scaffolds, bound with streptavidin and biotinylated IL4, were incubated with 20 μL of cell culture medium for 30 min in the incubator (37 °C, 5% CO₂) to improve the cell attachment. Cells were then seeded onto modified scaffolds, as described earlier. Media was collected every 2 days and entirely replaced with fresh complete medium (RPMI 1640 + 2% heat inactivated human serum + 1% penicillin streptomycin). Collected medium was centrifuged for 10 min at 400 g to remove dead cells and scaffold remnants, then stored at −80 °C. On day 7, conditioned media was collected, and scaffolds were placed in Trizol, snap-frozen in liquid nitrogen, then stored at −80 °C.

**Protein Secretion Analysis.** ELISA was conducted according to the manufacturers’ instructions to quantify the secretion of CCL18 and CCL22 (R&D Systems), known as M2 protein markers, in macrophage-conditioned media from cell-seeded scaffolds.

**RNA Extraction from Cell-Seeded Scaffolds and Explants.** After cell-seeded scaffolds and explants were thawed, 2–4 aluminum beads were added to the samples, then disrupted for three cycles of 10 s using a bead beater (Mini-Beadbeater-16, Biospec Products) or dismembrator (Sartorius, Gottingen, Germany). RNA was extracted using the RNeasy kit (QIAGEN) following the manufacturers’ instructions. The concentration of extracted RNA was measured using a Nanodrop ND1000 and was considered pure if the 260/280 ratio was above 1.8. The RNA concentration was quantified using the RNeasy kit (QIAGEN) following the manufacturers’ instructions to quantify the secretion of CCL18 and CCL22 (R&D Systems), known as M2 protein markers, in macrophage-conditioned media from cell-seeded scaffolds.

**Quantitative Reverse Transcription Polymerase Chain Reaction.** DNase treatment was conducted on thawed RNA using the DNase I, RNase-free kit following the manufacturer’s instructions (Thermo Fisher). Complementary DNA (cDNA) synthesis was performed using the High Capacity cDNA Reverse Transcriptase kit, according to the manufacturer’s instructions (Thermo Fisher). The SYBR Green PCR Master Mix (Applied Biosystems) was used to perform qRT-PCR to quantify the expression of the following genes for qRT-PCR and NanoString validation using 20 ng of cDNA per sample.
reaction: IL1B, VEGFA, MRC1, CCL22, and CCL18, as well as the housekeeping gene GAPDH. All primers were custom-designed and synthesized by Thermo Fisher (Supporting Information, S3). The ΔCt method was used to calculate the relative gene expression of the target probes normalized to GAPDH expression.

**Statistical Analysis.** Data are represented as mean ± standard error of the mean (SEM) and were analyzed in GraphPad Prism 8.0. Data were analyzed with either a one-way analysis of variance (ANOVA) or a Kruskal–Wallis test, based on whether the data exhibited a parametric or non-parametric distribution, respectively. A two-way ANOVA was used when two factors were compared within the experiments. Either the Tukey post-hoc or Dunn’s post-hoc multiple comparisons test or Kruskal–Wallis test, based on whether the data exhibited a parametric or non-parametric distribution, respectively. Comparisons were considered significantly different if \( p < 0.05 \).

**RESULTS**

**Biotinylation of Scaffolds and IL4.** Since the number of biotin molecules bound to each scaffold directly affects the number of bound avidin molecules, we first assessed how this property can be controlled by varying the FME of the biotinylation reagent. Biotinylation was tested for both glutaraldehyde-cross-linked and non-cross-linked scaffolds as glutaraldehyde fixation is often used to slow down the degradation of collagenous scaffolds. As expected, the moles of biotin conjugated to 0.1% glutaraldehyde-cross-linked gelatin scaffolds increased with increasing FME of the biotinylation reagent, with no further increase in biotin binding from 10 to 100 FME (Supporting Information, S4). There were no significant differences in biotinylation efficiency between non-cross-linked scaffolds and those cross-linked with 0.1% glutaraldehyde (Figure 2A). There were also no significant differences in the binding efficiency between scaffolds washed multiple times or those quenched with glycine, showing that washing effectively removed any residual glutaraldehyde from the scaffolds (Supporting Information, S5).

The HABA assay was not sensitive enough to determine the quantity of biotin bound to IL4 at concentrations less than several milligrams. Therefore, to confirm that increasing the FME of the conjugation reagent achieved the intended effect of increasing degree of the conjugation, a model fluorescent molecule, NHS-DyLight 350 (MW = 874 g/mol), was selected. As expected, increasing the FME of the DyLight 350 resulted in a significant increase in bound DyLight 350 per mol of IL4 (Figure 2B). However, since the DyLight 350 and biotinylation reagents utilize slightly different mechanisms and conditions, it is not possible to directly extrapolate these results to determine the extent of biotinylation.

**Binding of Avidin Variants to Biotinylated Scaffolds and Biotinylated IL4.** After achieving various degrees of biotinylated, cross-linked gelatin scaffolds, we then assessed the specificity of fluorescent CaptAvidin and streptavidin binding to biotin. Biotinylated, cross-linked scaffolds without any fluorescent avidin variant served as the negative control, while unbiotinylated, cross-linked scaffolds were associated with either DyLight 350-conjugated CaptAvidin or Alexa Fluor 488 streptavidin as a nonspecific adsorption control. Experimental scaffolds were then cross-linked with 0.1% glutaraldehyde, biotinylated with 1 FME of the biotinylation reagent, associated with either DyLight 350-conjugated CaptAvidin or Alexa Fluor 488 streptavidin, then imaged with both the negative control and nonspecific adsorption control scaffolds.
After confirming the specificity of CaptAvidin and streptavidin binding to biotinylated gelatin scaffolds, we investigated the effect of avidin control variant binding. In general, there was a dose-dependent effect of the avidin content on binding CaptAvidin or streptavidin to biotinylated scaffolds (Figure 2C,D). However, increasing the avidin content from 1 to 10 FME did not significantly increase CaptAvidin or streptavidin binding and actually caused a decrease in streptavidin intensity in the non-cross-linked scaffolds. Otherwise, cross-linking did not significantly affect CaptAvidin or streptavidin associated with biotinylated gelatin scaffolds (Figure 2E,F). Considering that we added an excess of avidin to the biotinylated scaffolds (Supporting Information, S7), it can be assumed that the amount of bound avidin is no more than the amount of bound biotin.

Release Kinetics of Biotinylated IL4 from Modified Scaffolds. The majority of biotinylated IL4 released from scaffolds occurred within the first 10 h, although detectable levels were released for up to 10 days (Supporting Information, S8). Surprisingly, there were no differences in release kinetics between scaffolds modified with either streptavidin or CaptAvidin when compared to the control with only adsorbed IL4 (no avidin variant) (Supporting Information, S8). For scaffolds modified with streptavidin, at the early time points, there was a trend toward decreasing IL4 release with increasing degree of biotinylation, but this effect was only significant at the 6 h time point (Supporting Information, S8).

We also conducted release studies in the presence of excess biotin to determine whether the free biotin would accelerate the release of biotinylated IL4, which has been previously demonstrated since biotinylated molecules have lower binding affinities compared to free biotin.20,27–29 The presence of excess biotin had no significant effect on the release profiles of biotinylated IL4 (Supporting Information, S9), except for the Ads IL4 (control) and 0.1 FME with CaptAvidin groups between 2 and 6 h (Supporting Information, S9). There was a significant increase in IL4 released from the Ads IL4 compared to the 10 FME with the streptavidin group but no other differences between groups (Supporting Information, S9). Free biotin did also accelerate the release of adsorbed IL4 at all timepoints in the unwashed group, which was prepared with biotinylated IL4 and biotinylated scaffolds but with no avidin variant and without washing (Supporting Information, S10).

Effects of Modified Scaffolds on the Macrophage Phenotype. We first verified that the biotinylation process did not affect the stimulatory properties of IL4 on macrophage activation (Supporting Information, S11), although it is important to note that non-biotinylated protein was not purified from these samples. Next, we investigated the ability of modified scaffolds to polarize macrophages toward the M2-like phenotype. Total protein secretion of the M2-like markers, CCL22 (Figure 3A) and CCL18 (Supporting Information,
was quantified in pooled conditioned media samples collected throughout the 2-week culture period. All scaffolds with IL4 generally resulted in higher secretion of CCL22 compared to the controls without IL4 at each time point (Figure 3B), but these differences were not always statistically significant (Supporting Information, S13). While scaffolds caused a trend toward increased CCL18 secretion compared to the blank and No IL4 controls on day 4, only the Ads IL4 and 10 FME + CaptAvidin groups were significantly different from the controls by day 14 (Supporting Information, S12).

Gene expression at the 14 day time point was assessed to measure the sustained maintenance of phenotype modulation. There was a trend toward increased expression of the M2-associated marker CCL22 in groups with biotinylated IL4 compared to the blank and No IL4 controls, with significantly higher gene expression in the 10 FME + streptavidin and 1 FME + CaptAvidin groups (Figure 3C). Increasing the degree of biotinylation from 1 FME to 10 FME when used with CaptAvidin caused a significant decrease in the expression of CCL22 (Figure 3C). All groups with biotinylated IL4, except for 1 FME + streptavidin, expressed significantly higher levels of the M2-like marker CCL18 compared to the blank and No IL4 controls (Figure 3D), while there were no significant differences between groups for the M2-like marker MRC1 (Figure 3E). There were no significant differences between groups in the expression of the M1 markers IL1B or VEGFA (Figure 3F,G).

Binding of Fluorescent Streptavidin and Biotinylated IL4 under Flow. To understand the in situ loading potential of biotin–avidin interactions, such as for bioactivation via systemic injection, Fstrep was introduced to biotinylated scaffolds under flow conditions (14.3 mL/min and shear stress = 5 Pa) (Figure 4A) and visualized by an increase in mean fluorescent intensity (Figure 4B). After 30 min of streptavidin exposure, 1 FME and 10 FME biotin scaffolds bound significantly more streptavidin compared to 0.1 FME with no
biont scaffolds (Figure 4C). The 0.1 FME bound slightly more Fstrep compared to the no biont scaffolds ($p = 0.0559$) (Figure 4C).

Following loading of biontlated scaffolds with streptavidin and biontylated IL4 under flow, macrophages were cultured under static conditions to measure bioactivity. Total secretion of CCL22 after 7 days was significantly higher for all IL4-containing groups (Ads IL4, static, and flow) compared to the groups without IL4 (blank and No IL4) (Figure 5A). Scaffolds functionalized with IL4 under flow resulted in higher levels of total secretion of CCL22 compared to all other groups (Figure 5A). Additionally, the flow group resulted in significantly more CCL22 on days 1, 5, and 7, peaking at day 5 (Figure 5B). Results from qRT-PCR showed an upregulation of all M2-associated genes (CCL22, CCL18, and MRC1) in the flow group (Figure 5C–E). The M1-associated markers (IL1B and TNFA) were expressed at higher levels in groups without IL4 (blank and No IL4) compared to the IL4-containing groups (Ads IL4, static, and flow) (Figure 5F,G).

We also measured expression of genes associated with oxidative degradation via reactive oxygen species (ROS) (NFKB1 and CYBB) or enzymatic degradation via gelatinases (MMP2 and MMP9). While cross-linking with gluteraldehyde had no impact on macrophage polarization, it significantly decreased expression of NfkB, MMP2, and MMP9 (Supporting Information, S14). For oxidative degradation, the No IL4 group caused an increased expression of NFKB1 compared to the Ads IL4 and static groups (Figure 5H) and increased expression of CYBB compared to all other groups (Figure 5I). For enzymatic degradation, groups with IL4 (Ads IL4, static, and flow) caused significantly lower expression of MMP2 and MMP9 compared to non-IL4 groups (blank and No IL4) (Figure 5J,K). Additionally, MMP9 expression was significantly lower in the flow group compared to all other groups (Figure 5K).

**DISCUSSION**

The biont–avidin affinity conjugation system has been used in a variety of biomaterial design strategies and tissue integration strategies, but the extent to which this system can be used to impart immunomodulatory activity to biomaterials has not been previously investigated. The goal of this study was to investigate how biont density and avidin variant affect the loading and bioactivity of the immunomodulatory cytokine, IL4, on a model porous gelatin scaffold. We found that modulating the degree of biontylaion and the avidin variant controls bioactivity of IL4 in vitro. Yet, biont–avidin interactions did not strongly influence the release of IL4 in this system, which may result from a large degree of nonspecific adsorption of both nonbiontylated and biontylated IL4, described in more detail below. Nonetheless, increasing degrees of biontylaion did lead to increased cumulative CCL22 secretion, a key M2 marker, of primary macrophages in vitro, highlighting the capability to leverage biont–avidin interactions to modulate the macrophage phenotype. Moreover, we showed proof-of-concept for the in situ loading of biontylated scaffolds under conditions of high shear stresses, suggesting that the system could potentially be used to direct immunomodulatory cytokines to biontylated biomaterials in vivo. We used biontylated cross-linked Gelfoam and biontylated IL4 as a model biomaterial and protein, respectively, but the modular nature of commercially available biontylaion reagents makes this a versatile strategy to add biofunctionality to virtually any biomaterial.

The response of host macrophages to implanted biomaterials is a major determinant of their success or failure, so the need for strategies to impart immunomodulatory activity to biomaterials is critical. Incorporation of IL4 is a widely used strategy in various recent studies because it has demonstrated to be effective in modulating the macrophage phenotype and the FBR, both in vitro and in vivo. For example, Tolouei et al. used a magnetically sensitive hydrogel to achieve delayed IL4 delivery, while Hachim et al. used a polymeric coating to control the sustained release of IL4 from scaffolds, resulting in reduced fibrous encapsulation in a mouse abdominal subcutaneous pocket model. Controlling the temporal delivery of IL4, and possibly other immunomodulatory cytokines, is an important challenge in order to improve durability and integration of implanted biomaterials. Using biont–avidin interactions as a modular platform to attach and release immunomodulatory proteins to biomaterials would be particularly beneficial for complex biomaterials, which are not amenable to polymer coatings or other traditional methods of drug delivery. A modular system with many reagents available for bioconjugation to different proteins or drugs, with the potential for timed release, is important because optimal tissue healing and biomaterial integration require sequential presentation of multiple macrophage phenotypes, although the optimal timing is not yet known. In addition, biont can be attached to polymers, proteins, and even cells, without affecting their bioactivity to allow for specific binding of biontylated proteins only. Finally, the biont–avidin system has been used clinically to facilitate labeling and tracking of intravenously delivered therapeutics, highlighting the clinical potential of biont–avidin-based biomaterials.

Gelfoam scaffolds functionalized with biont were capable of binding streptavidin and CaptAvidin, but different levels of biont–density did not significantly affect the binding of these proteins. Interestingly, the release of IL4 did not differ between scaffolds with and without avidin (adsorbed control). This can be attributed to the fact that the IL4 was not purified after biontylaion. Even though scaffolds were thoroughly washed in an attempt to remove unbiotinylated IL4, it appears likely that unbiotinylated IL4 still remained on the scaffold and obscured biont–avidin-mediated release kinetics. Additionally, nonspecific adsorption of biontylated IL4 to scaffolds with and without avidin may have also occurred. The presence of excess biont, which is known to trigger the release of lower affinity biont conjugates, did not affect release, except for the adsorbed, unwashed group that did not contain either avidin protein. The triggered release of IL4 by the excess biont in this group is likely explained by the Vroman effect, in which small molecules displace larger ones on a biomaterial's surface. Excess biont did not displace detectable levels of biontylated IL4 from washed scaffolds regardless of whether they contained an avidin protein, even though excess biont is well known to cause release of biont conjugates from a wide array of substrates. The fact that the adsorption forces were stronger than biont–avidin interactions further supports the conclusion that nonspecific adsorption of unbiotinylated IL4 obscured any effects of biont–avidin interactions. Indeed, other studies have similarly reported surprisingly high adsorption capacity of Gelfoam, with one study reporting that Gelfoam scaffolds had to be degraded with trypsin in order to completely release adsorbed peptides. Another study...
showed high nonspecific adsorption of IL4 to agarose substrates. A slight reduction in release of IL4 over the first day from scaffolds with increasing biotinylation and streptavidin may result from diminished nonspecific IL4 adsorption by surface bound avidin, or from biotin–avidin interactions, or both. It is also possible that the extent of scaffold biotinylation influenced nonspecific adsorption capacity. Future studies should thoroughly investigate the influence of protein adsorption capacity on the effects of biotin–avidin interactions on the release of purified biotinylated proteins.

Even though biotin–avidin interactions did not influence release profiles of IL4, they clearly enhanced the immunomodulatory capacity of the scaffolds because macrophages secreted more CCL22 when cultured on scaffolds containing both biotinylated IL4 and CaptAvidin compared to adsorbed IL4 controls. The immunomodulatory function of IL4 is exerted by binding IL4 to its receptor, IL4R, on the cell surface, which does not require release of IL4 from the scaffold. Therefore, although the release of IL4 could not be adequately controlled with the biotin–avidin system with Gelfoam, it did allow modulation of effective scaffold functionalization. Future studies should be directed toward understanding how both material properties and biotin–avidin interactions can be manipulated to control presentation and release of biotinylated proteins, perhaps by decreasing adsorptive properties of the material or varying the length of the spacer arm between biotin and the protein.

Despite the visible effects of IL4-functionalized scaffolds on macrophage polarization, the actual dose of biotinylated IL4 bound to the scaffolds prepared with streptavidin could not be directly quantified. Unfortunately, attempts at semi-quantitative analysis following immunofluorescent staining or indirect ELISA of wash solutions were not successful due to sensitivity issues. Future studies should focus on measuring biotinylination efficiency and thoroughly characterizing the binding and release of biotinylated proteins using sensitive techniques, for example, via radiolabeling techniques. These experiments would also address the surprising decrease in Fstrep binding upon increasing biotinylation from 1 to 10 FME in non-cross-linked scaffolds.

In this study, we also executed proof-of-concept work that streptavidin and biotinylated IL4 could be loaded onto biotinylated scaffolds via flowing solutions over the scaffolds. The potential to load a biomaterial in vivo is particularly important in order to modulate the macrophage phenotype around the implanted biomaterial, in order to promote a delayed M2 phase without inhibiting the early M1, inflammatory phase. Previously, Hoya et al. showed that biotinylated drugs could be targeted to biotinylated cells in vivo by biotinylating the renal vasculature of a rabbit, injecting avidin, then following up with fluorescein-biotin, where over 80% of the treated tissue retained its biotinylated “drug.” Here, we opted for imposing a relatively high shear stress of 5 Pa, which naturally occurs on the surface of heart valves, in order to demonstrate the loading efficiency, even under extreme conditions. The efficient loading under these conditions suggests the potential to load implanted materials that are directly exposed to high blood flows, like cardiovascular implants of heart valves or blood vessels. In our study, an interesting finding was the increase in the response of macrophages to scaffolds loaded with streptavidin under flow compared to those loaded statically. In the flow studies, a higher mass of biotinylated IL4 was employed to account for potential decrease in binding; therefore, it seems likely that more IL4 bound or adsorbed to the scaffolds despite the high flow conditions, leading to enhanced effects on macrophage polarization.

The gelatin scaffold used in this study was selected as a model material, representing often-used collagenous scaffolds. The scaffolds were cross-linked with glutaraldehyde to prevent rapid degradation, as often employed in clinical applications, such as bioprosthetic heart valves. In the future, in vivo studies should elucidate the effect of scaffold functionalization with IL4 or other immunomodulatory cytokines via biotin–avidin on implant integration and healing over time, by investigating the macrophage phenotype and tissue formation around the implanted scaffold, preferable for both degradable and non-degradable materials.

**CONCLUSIONS**

We identified that biotin–avidin interactions can be leveraged to impart immunomodulatory activity to porous scaffolds. Modulating the degree of biotinylation controlled the binding of two different avidin variants, and biotinylated scaffolds could be targeted by streptavidin and biotinylated IL4 under flow. We showed that increasing degrees of biotinylation of the scaffolds led to increased M2-like macrophage polarization over time. This suggests the potential use of biotin–avidin to modulate the macrophage phenotype in vitro and potentially in vivo. Future studies should focus on establishing fine-tuned control over biotin–avidin interactions and further investigate the binding and release mechanisms in order to optimize release of macrophage-modulating cytokines for different clinical applications.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomaterials.1c01190. Calculations of shear stress for flow experiments; purity of isolated monocytes for flow studies (flow cytometry); primers used for qRT-PCR; upper and lower range of biotinylation of porous gelatin scaffolds (HABA assay); effect of glycine quenching on cross-linked gelatin scaffolds; specificity of binding of fluorescent avidin variants to cross-linked and biotinylated scaffolds; theoretical amount of the bound avidin variant to biotinylated scaffolds; release of biotinylated IL4 from modified scaffolds in the absence of excess biotin; release of biotinylated IL4 from washed modified scaffolds; effect of biotinylating IL4 on macrophage polarization; protein secretion of CCL18 at select timepoints; protein secretion of CCL21 at select timepoints; and effect of glutaraldehyde cross-linking on macrophage polarization (PDF)

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Notes
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