Antibody-mediated trapping in biological hydrogels is governed by sugar-sugar hydrogen bonds

Jennifer L. Schiller[a], Mary M. Fogle[a], Olivia Bussey[a], William J. Kissner[b], David B. Hill[b,c], Samuel K. Lai[a,d,e]*

[a]Division of Pharmacoengineering and Molecular Pharmaceutics, Eshelman School of Pharmacy; [b]Department of Physics and Astronomy; [c]Marsico Lung Institute/CF Center; [d]UNC/NCSU Joint Department of Biomedical Engineering; [e]Department of Microbiology & Immunology; University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

*Corresponding author:
Samuel K. Lai
Division of Pharmacoengineering and Molecular Pharmaceutics
University of North Carolina at Chapel Hill
Marsico 4213, 125 Mason Farm Road
E-mail: lai@unc.edu
Homepage: https://pharmacy.unc.edu/research/faculty-labs/lai-research-group/

Abstract

N-glycans on IgG and IgM antibodies (Ab) facilitate Ab-mediated crosslinking of viruses and nanoparticles to the major structural elements of mucus and basement membranes. Nevertheless, the chemical moieties in these biological hydrogel matrices to which Ab can bind remain poorly understood. To gain insights into the chemistries that support Ab-matrix interactions, we systematically evaluated IgG- and IgM-mediated trapping of nanoparticles in different polysaccharide-based biogels with unique chemical features. In agarose, composed of alternating D-galactose and 3,6-anhydro-L-galactopyranose (i.e. hydroxyl groups only), anti-PEG IgM but not anti-PEG IgG trapped PEGylated nanoparticles. In alginate, comprised of homopolymeric blocks of mannuronate and guluronate (i.e. both hydroxyl and carboxyl groups), both IgG and IgM trapped PEGylated nanoparticles. In contrast, chitosan, comprised primarily of glucosamine (i.e. both hydroxyl and primary amine groups), did not facilitate either IgG- or IgM-mediated trapping. IgG-mediated trapping in alginate was abrogated upon removal of IgG N-glycans, whereas IgM-mediated trapping was eliminated in agarose but not alginate upon desialylation. These results led us to propose a model in which hydrogen bonding between carboxyl and hydroxyl groups of glycans on both Ab and matrix facilitates Ab-mediated trapping of pathogens in biogels. Our work here offers a blueprint for designing *de novo* hydrogels that could harness Ab-matrix interactions for various biomedical and biological applications.

Key words: biological hydrogels, glycosylation, antibodies, hydrogen bonds

1 Introduction

Biological hydrogels (biogels) are ubiquitous in most living systems: red algae produce agarose to fortify their cell walls [1]; insects build durable, strong exoskeletons from chitin [2]; and bacteria secrete alginate as part of their protective biofilms [3]. In humans, secreted mucus lubricates and protects against invading pathogens, while extracellular matrices provide structural support for cell scaffolding [4-6]. Structurally, these diverse biogels are comprised of entangled and/or crosslinked biopolymers, creating a matrix with well defined mesh spacings [7]. The barrier properties of biogels stem at least in part from steric occlusion of the diffusion of particulates that are similar if not larger in size than the pores present in the biogel. Biogels comprised of charged polymers can also directly capture much smaller particles electrostatically [5, 8-12]. Since the matrix constituents of the biogels dictate both their physical properties and precise biological functions, their biochemistries are typically well conserved and do not readily change over time. This precludes biogels from dynamically tuning their barrier properties against diverse species with any molecular specificity.

Previously, our group has demonstrated that antibodies (Ab) can act as third-party molecular crosslinkers that immobilize specific nanoparticulate species in native biogels, including several types of mucus and basement membrane [8, 13-17]. In particular, the Fab domains of IgG and IgM can evolve to bind entities of interest with high specificity, ranging from viruses and nanoparticles to highly motile bacteria, and immobilize them through Fc-matrix bonds. Trapping in biogels prevents these species from permeating the biogel barrier and reaching target cells over a time scale commensurate with natural mucus clearance [16, 18-22]. For example, non-neutralizing anti-HSV IgG trapped Herpes Simplex Virus in cervicovaginal mucus and blocked vaginal herpes transmission *in vivo* [13]. Basement membrane treated with anti-LPS IgG stalled the passage of *Salmonella typhimurium* [8]. Topical administration of ZMapp^{TM}, a cocktail of three mAbs against Ebola, effectively trapped Ebola in human airway mucus and facilitated the clearance of the virus from the mouse lung within 30 minutes [17].

N-glycans on IgG and IgM appear to play a critical role in facilitating Ab-mediated trapping in various biogels [8, 13]. However, the precise chemistries on biogels that support Ab-matrix interactions remain poorly understood. The biogels that were found to facilitate Ab-mediated trapping, such as mucus and laminin, all contain an abundance of complex glycans [8, 14, 15, 17, 23], including high levels of sialic acid, a sugar possessing both N-acetyl and carboxyl groups [6, 24]. In comparison, in collagen IV gels that failed to facilitate effective IgG-mediated trapping [8], there is a much lower level of glycosylation, with only a single galactose and/or glucose at any given glycosylation site [25]. These observations led us to hypothesize that complex glycans, and more specifically the carboxyl groups on these glycans, represent the key chemical moiety on matrix constituents facilitating Ab-biogel interactions.

To test this hypothesis, we investigated IgG- and IgM-mediated trapping in polysaccharide-based biogels with simple, well-defined structures: agarose, chitosan, and alginate (Fig. 1). Each biogel allowed for the isolation of a specific functional group to study its impact on Ab-mediated trapping. Agarose, a disaccharide comprised of alternating D-glactose and 3,6-anhydro-L-galactopyranose, contains only hydroxyl and ester functional groups (Fig. 1A) [26]. Chitosan (Fig. 1B) is composed predominantly of glucosamine, with some small quantity of N-acetyl glucosamine, which possess amine groups in addition to hydroxyl groups [27]. Alginate (Fig. 1C) is composed of homopolymeric blocks of mannuronate, guluronate, or alternating residues, all of which possess carboxylic acids in addition to hydroxyl groups [28]. Quantifying IgG- and IgM-mediated trapping in these biogels thus allows us to systemically investigate the influence of hydroxyl, amine and carboxyl groups along the matrix on interactions with both Ab.

2 Materials and Methods

2.1 Materials

Growth factor-reduced Matrigel and high concentration laminin/entactin were obtained from Corning. Sodium alginate, lyophilized mouse collagen IV, D(+)-glucono-δ-lactone (GDL), and calcium carbonate ($CaCO_3$) were purchased from Sigma Aldrich. Chitosan was obtained from Acros Organics. β-glycerophosphate (BGP) was obtained from Alfa Aesar.

2.2 Preparation of PEG-coated nanoparticles

To produce PEGylated nanoparticles (PS-PEG), we covalently modified 40 nm or 100 nm fluorescent, carboxyl-modified polystyrene beads (PS-COOH; Invitrogen) with 5 kDa methoxy polyethylene glycol amine (PEG; Sigma) via a carboxyl-amine reaction, as published previously [29, 30]. Particle size and ζ-potential were determined by dynamic light scattering and laser Doppler anemometry, respectively, using a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA). Size measurements were performed at 25 °C at a scattering angle of 90°. Samples were diluted in 10 mM NaCl solution, and measurements were performed according to instrument instructions. PEG conjugation was also confirmed by a near-neutral ζ-potential [29]. Dense PEG grafting (>1 PEG/nm^2) was further verified using the fluorogenic compound 1-pyrenyldiazomethane (PDAM) to quantify residual unmodified carboxyl groups on the polystyrene beads [30]. 200, 1000, and 2000 nm PS-COOH and 200 nm amine-conjugated fluorescent nanoparticles (PS-NH_2; Invitrogen) were also used.

2.3 Antibodies

Anti-PEG IgG_1 (CH2076, Silver Lake Research) and anti-PEG IgM (AGP4, IBMS) were used as test Ab and anti-Biotin IgG_1 (Z021, Thermo Fisher) and anti-Vancomycin IgM (2F10, Santa Cruz) were used as control Ab. CH2076 was deglycosylated with rapid, non-reducing PNGase F (New England Biolabs) according to the manufacturer's instructions. Deglycosylation was confirmed by SDS-PAGE gel with Coomassie stain and lectin-ELISA with biotinylated concanavalin A (B-1005, Vector Labs) and HRP-

conjugated anti-biotin IgG (033720, Life Technologies). IgM was desialylated overnight with α2-3,6,8,9 Neuraminidase A (New England Biolabs) with 3 µL enzyme per 1 µg antibody at 37°C. Desialylation was confirmed with lectin-ELISA with biotinylated elderberry bark lectin (B-1305, Vector Labs), biotinylated concanavalin A, and HRP-conjugated anti-biotin IgG. Due to the overnight incubation at 37°C required to fully desialylate IgM and concerns it might denature IgM or otherwise reduce binding affinity to either PEG or the biogel matrix, we compared the trapping potency of desialylated IgM native IgM that was also incubated overnight at 37°C.

2.4 Preparation of Biogels

Generally, biogels were prepared by adding a mixture of the following to a custom-made micro-volume (~10 µL) glass chamber slide: (i) biogel, (ii) diluent, (iii) polymerization agents, (iv) fluorescent 2000 nm PS-COOH or 40 nm or 100 nm PS-PEG beads, and (v) different Ab (IgG concentration was 10 µg/mL and IgM concentration was 5 µg/mL, in line with previous work). The mixture was incubated for 10 minutes at a temperature determined experimentally before sealing and an additional 5 minutes of incubations (Table 1). To account for later addition of antibodies, control gels were made with at least 10% PBS. Agarose gels, as when preparing such gels for electrophoresis, was supersaturated at a high temperature, then slowly cooled on a hot plate until about 45°C as measured by thermometer. At this temperature, we did not worry about short-term denaturing of antibodies, especially as the gel cooled as it set. Chitosan is only soluble in acid, so we initially dissolved it in 1.2 M lactic acid. Addition of the cross-linker, BGP, neutralized it to ~pH 7. All chitosan reagents were kept chilled until incubation. Alginate is well known to be crosslinked by calcium ions. For slow release of calcium, we used equimolar quantities $CaCO_3$ and GDL, a weak acid. This acid did not affect the overall neutral pH of the gel.

2.5 High-resolution multiple particle tracking

The trajectories of the fluorescent particles were recorded using an EMCCD camera (Evolve 512; Photometrics, Tucson, AZ) mounted on an inverted epifluorescence microscope (AxioObserver D1; Zeiss,

Thornwood, NY), equipped with an Alpha Plan-Apo 100x/1.46 NA objective, environmental (temperature and CO_2) control chamber and an LED light source (Lumencor Light Engine DAPI/GFP/543/623/690). 20 s videos (512 x 512, 16-bit image depth) were captured with MetaMorph imaging software (Molecular Devices, Sunnyvale, CA) at a temporal resolution of 66.7 ms and spatial resolution of 10 nm (nominal pixel resolution 0.156 μm/pixel). The tracking resolution was determined by tracking the displacements of particles immobilized with a strong adhesive, following a previously described method [31]. Particle trajectories were analyzed using a neural network software as described previously [32, 33]. Sub-pixel tracking resolution was achieved by determining the precise location of the particle centroid by light-intensity-weighted averaging of neighboring pixels. Trajectories of $n \geq 40$ particles per frame on average (corresponding to $n \geq 100$ total traces) were analyzed for each experiment. The coordinates of particle centroids were transformed into time-averaged mean squared displacements (MSD), calculated as $\langle \Delta r^2(\tau) \rangle = [x(t + \tau) - x(t)]^2 + [y(t + \tau) - y(t)]^2$ (where τ = time scale or time lag), from which distributions of MSDs and effective diffusivities (D_{eff}) were calculated, as previously demonstrated [29]. Mobile particles were defined as those with $D_{eff} \geq 10^{-1}$ μm^2/s at τ = 0.2667 s (this τ corresponds to a minimum trajectory length of 5 frames) [9, 29].

2.6 Rheological Characterization of Biogels

Macrorheological properties of biogels were measured by performing amplitude sweeps and frequency experiments on a TA Discovery Hybrid Rheometer 3 (TA Instruments, New Castle, Delaware) with a 20 mm diameter 10 cone as described previously. Matrigel, laminin, and collagen IV were preformed in a microcentrifuge tube at 37°C for 2 hours as prepared previously [8], while agarose and alginate were gelled *in situ* on the rheometer for 10 minutes [34]. Experiments were performed three times and did not contain fluorescent nanoparticles or antibodies, but otherwise resembled gels used in particle tracking experiments. Three separate gels were loaded. All analyses were performed at the temperature listed in Table 1 and employed a solvent trap to minimize sample dehydration. Once

loaded, the following assays were run on each sample: 1) 0.1 and 10 Hz stress sweeps ranging from 0.01 Pa to 10 Pa to ascertain the linear viscoelastic regime (LVR); and 2) 0.01 – 100 Hz frequency sweeps at stresses below the nonlinear threshold, yielding macroscopic linear moduli. Macroscopic rheological data were analyzed via TA Trios software. Complex viscosity (η*) values measured by amplitude sweeps were directly reported by Trios.

2.7 ELISA

Half-area 96-well plates (Corning, 3693) were coated with 50 µg/mL 10k DSPE-PEG in PBS. Plates were blocked with 5% non-fat milk in PBS for 30 minutes. IgG anti-PEG or IgM anti-PEG was diluted to 500 ng/mL in 1% milk in PBS, 1% milk in 500 mM NaCl, or 1% milk in 1.2 M lactic acid and 132.3 mg/mL BGP, then sequentially diluted two-fold. Either 1:10,000 Goat anti Mouse IgG, HRP Superclonal (A28177, Invitrogen) or 1:10,000 Goat anti-Mouse IgM (Heavy chain), HRP (62-6820,Invitrogen) in 1% milk was used as secondary, as appropriate. Plates were developed with 1 Step Ultra TMB Substrate Solution (Thermo Scientific Pierce) and the reaction halted with 1 N HCl. Plates were read at 450 nm and 570 nm on a SpectraMax M2 Microplate Reader (Molecular Devices). In a similar assay, anti-PEG IgG and deglycosylated anti-PEG IgG were used as the primary antibodies.

2.8 Software

Chemicalize (https://chemicalize.com/, accessed June, 2019, developed by ChemAxon (http://www.chemaxon.com)) was used to draw structures and predict pKa values as shown in Fig 1. ChemDraw 18.0 was used to model H-bonds as seen in Fig 7. Adobe Illustrator was used to draw the IgG and IgM glycoengineering schematics in Figs 3 and 5. GraphPad Prism was used to produce all other figures.

2.9 Statistics

All statistical analysis was performed in GraphPad Prism and was two-sided. MSD data were log-log-transformed and compared within groups using a repeated-measures (RM) two-way analysis of

variance (ANOVA) and post hoc Šidák test. Average D_{eff} and % mobile were compared with ANOVA and subsequent Šidák tests. In all analyses, family-wide significance level α=0.05. Error bars and ± represent standard error of the mean (SEM) or confidence interval (CI), as listed in the caption. An individual experiment was defined as a discrete sample preparation (e.g. one slide). Multiple videos were taken of each gel to ensure imaging throughout the sample and sufficient numbers of beads captured.

2.10 Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

3 Results and Discussion

3.1 PEGylated nanoparticles are mobile in biogels

We first sought to ensure that the biogels possess sufficient rigidity to facilitate antibody-mediated trapping. By relying on a combination of prior literature and experimentation, we formulated each of the biogels to possess (1) pore sizes sufficiently large to allow rapid diffusion of 40-100 nm PEGylated nanoparticles and (2) sufficient matrix rigidity to trap particles by either electrostatic interaction (with charged particles) or steric obstruction (with larger microspheres; Suppl. Fig. 1). Both agarose and chitosan allowed rapid diffusion of 100 nm PEG nanoparticles, reminiscent of the rapid diffusion of similarly-sized HIV VLPs we have previously observed in cervicovaginal mucus [35]. However, direct binding of 100 nm COOH or NH_2 beads to both matrices by direct electrostatic interactions or hydrogen bonding did not occur, and we instead inferred sufficient matrix rigidity based on immobilization of larger 1000-2000 nm COOH beads (Suppl. Fig. 1). For alginate, at concentrations that immobilized 1000 nm COOH beads, 100 nm PEG nanoparticles were not readily mobile. Reduction of either crosslinker or monomer concentration resulted in a gel that failed to set; as a result, 40 nm PEG beads (slightly larger than a poliovirus) were used instead. Due to differences in nanoparticle size and thus their inherent diffusivity, as well as differences in biogel composition, the diffusivities of

nanoparticles and distributions thereof cannot be directly compared between biogels; however, the impact of various treatments (e.g. application of antibodies) can be assessed within each biogel.

3.2 IgG mediates trapping of nanoparticles in alginate but not agarose or chitosan

Our previous work demonstrated that in both fresh, native mucus and Matrigel (2.2 mg/mL), 10 μg/mL of anti-PEG IgG was sufficient to effectively immobilize PEG-coated nanoparticles substantially smaller than the pore size of the matrix [8]. When treated with 10 μg/mL IgG (Fig. 2), both agarose and chitosan gels failed to trap PEG-coated nanoparticles. There was no appreciable difference in the logarithmic distribution of effective diffusivity ($\log(D_{eff})$; Fig. 2A) or in the ensemble averaged mean squared displacement (<MSD>) of PEG-coated nanoparticles between untreated or anti-PEG IgG-treated agarose gels (p=0.2912, Fig 2B.) The fraction of mobile PEG particles was ~56% [41%-71%] in native agarose vs. ~54% [41%-67%] in anti-PEG IgG treated agarose (p=0.9999; Fig. 2A). The average effective diffusivities ($<D_{eff}>$; measured at 0.267s throughout) were similarly unchanged (~0.12 [95% CI: 0.062-0.23] $\mu m^2/s$ in native vs. ~0.10 [0.043-0.23] $\mu m^2/s$ IgG-treated agarose, p=0.7951, Suppl. Fig. 2A). Likewise, the $\log(D_{eff})$ distributions of PEG nanoparticles in native vs. IgG-treated chitosan gels were virtually identical (Fig 2C), with ~100% of particles mobile [99.5%-100%] in native vs. ~96% [84%-100%] in IgG-treated chitosan (p=0.7632), and no appreciable difference in the <MSD> of PEG-coated nanoparticles in either native vs. IgG-treated chitosan (p=0.0952, Fig. 2D). The $<D_{eff}>$ of PEG nanoparticles were ~0.41 [0.29-0.58] $\mu m^2/s$ in native vs. ~0.31 [0.20-0.48] $\mu m^2/s$ in IgG-treated chitosan, p=0.8830; Suppl. Fig. 2A).

Unlike agarose and chitosan, anti-PEG IgG substantially reduced the mobility of PEG-coated nanoparticles in alginate. The mobile fraction of PEG nanoparticles was reduced from ~94% in native alginate [92%-96%] to ~24% [12% to 37%] in alginate treated with anti-PEG IgG (p<0.0001; Fig. 2E). Anti-PEG IgG substantially decreased the <MSD> of PEG-coated nanoparticles in alginate across all time

scales evaluated compared to native alginate without IgG ($p<0.0001$; Fig. 2F). Anti-PEG IgG reduced the $\langle D_{eff} \rangle$ ~5-fold, from ~0.28 [0.24-0.33] $\mu m^2/s$ to ~0.051 [0.032-0.080] $\mu m^2/s$ ($p=0011$, Suppl. Fig. 2A).

Previously, we discovered that removing Fc N-glycans abrogated the ability of IgG to mediate trapping in mucus [13] and basement membrane [8]. We hypothesized the same would be true in these biogels. We deglycosylated anti-PEG IgG with PNGase F as previously described (Fig. 3A; Suppl. Fig. 3). Deglycosylated IgG indeed failed to mediate appreciable trapping of PEG-coated nanoparticles in alginate (Fig. 3B-C, Suppl. Fig. 2B). Specifically, ~97% [92.9%-100%] of the PEG-coated nanoparticles were classified mobile in alginate treated with deglycosylated anti-PEG IgG ($p<0.0001$ compared to native IgG; Fig 3B) with much greater <MSD> than that in alginate gel treated with native anti-PEG IgG ($p>0.0001$; Suppl. Fig. 2B). The $\langle D_{eff} \rangle$ of PEG-coated nanoparticles in alginate with deglycosylated IgG was over 30-fold greater than that in alginate treated with native IgG ($p<0.0001$, Fig. 3C).

3.3 IgM mediates trapping in both alginate and agarose gels

We previously showed that IgM exhibited greater trapping potency than IgG [8, 14]. We next evaluated whether anti-PEG IgM can similarly mediate trapping of PEG-coated nanoparticles in our model biogels (Fig. 4). Unlike IgG, IgM facilitated trapping of PEG-coated nanoparticles in agarose, as reflected by the distinct distribution of the effective diffusivities of individual particles (Fig. 4A), with a >50% drop in the mobile fraction of nanoparticles, from ~56% [41%-71%] to ~26% [19%- 33%] ($p<0.0001$, Fig. 4A). Anti-PEG IgM reduced the <MSD> four to eight-fold across all time scales measured ($p<0.02$, Fig 4B); the $\langle D_{eff} \rangle$ was reduced almost 6-fold, from ~0.12 [0.062-0.23] $\mu m^2/s$ to ~0.021 [0.014-0.034] $\mu m^2/s$ ($p<0.0001$, Suppl. Fig. 2C).

Similar to IgG in chitosan gels, IgM in chitosan failed to mediate any appreciable change in the mobility of PEG-coated nanoparticles, as reflected by the virtually identical $\log(D_{eff})$ distributions, with nearly 100% of beads classified as mobile [99.5%-100%] in untreated vs. [99.7%-100%] in IgM-treated chitosan gels ($p=0.7632$; Fig. 4C). There was likewise no difference in <MSD> vs. time scale plots (Fig. 4D)

and <D_{eff}> (~0.41 [0.29-0.58] $\mu m^2/s$ in untreated vs. ~0.37 [0.34-0.41] anti-PEG IgM-treated chitosan, p=0.9880; Suppl. Fig. 2C).

Finally, IgM mediated highly effective trapping of PEG nanoparticles in alginate, reducing the mobile fraction from ~94% [92%-96%] to ~13% [6%-20%] (p<0.0001, Fig. 4E) and the <MSD> across all time scales (p<0.0001, Fig. 4F). The <D_{eff}> was reduced by ~30-fold from 0.28 [0.24-0.33] $\mu m^2/s$ to 0.0094 [0.0071-0.012] (p<0.0001, Suppl. Fig. 2C). Overall, IgM was more potent than IgG in trapping PEG-nanoparticles in alginate, with <Deff> reduced by ~10-100-fold with IgM vs. ~8-20-fold with IgG.

One of the major differences between IgG and IgM, other than the number of glycosylation sites, is the type of glycosylation. Only ~7-20% of IgG_1 are sialylated [36-39], whereas IgM have ~30 sites that are ~80% sialylated *in vivo* [40-42]. We thus sought to test if sialic acid was partly responsible for IgM's potency in facilitating trapping of nanoparticles in alginate and particularly in agarose gels. Sialic acid was removed from IgM with neuraminidase A, leaving the remainder of the glycan intact (Suppl. Fig. 3D; Fig. 5A). Desialylated (desialy.) IgM was less potent than native IgM in trapping nanoparticles in agarose: mobility of PEG-coated nanoparticles increased from ~21% [12%- 31%] in native IgM- to ~39% [28%-50%] in desialy. IgM-treated agarose (p=0.01; Fig. 5B), MSD increased by 4-9-fold across all time scales tested (p=0.0483; Fig. 5C), and the <D_{eff}> increased from ~0.024 [0.015-0.039] to ~0.059 [CI:0.032-0.11] $\mu m^2/s$ (p=0.023; Suppl. Fig. 2D). Interestingly, desialy. anti-PEG IgM trapped PEG nanoparticles just as well in alginate as native IgM; the mobile fractions (Fig. 5D), <MSD> (Fig. 5E), and <D_{eff}> (Suppl. Fig. 2D) were all virtually unchanged between the two groups.

3.4 Biogel rheology and ionic strength do not explain their differential trapping phenomenon

Given the distinct chemistries and preparations of the different biogels, they inherently possessed different bulk rheological properties. To confirm our microscopy findings were not directly correlated to simple differences in the overall biogel viscoelasticity, we characterized the bulk rheology of the various biogels using cone-and-plate rheometry (Suppl. Fig. 4). Our preparation of agarose had an

elastic modulus of ~40 Pa (frequency 1 Hz), whereas alginate's was over twice that at ~100 Pa. Matrigel and laminin, which we have previously shown to facilitate both IgG- and IgM- mediated trapping, were much softer, with elastic moduli of ~3 Pa. Thus, the inability for IgG to mediate trapping in agarose was not due to inadequate G' or G" of the agarose gel, and the ability for antibody-mediated trapping does not appear to depend on a gel's bulk rheology.

Ionic strength negatively correlates with electrostatic trapping of charged molecules in charged biogels [43]. To create a chitosan gel, we dissolved it in 1.2 M lactic acid and neutralized with 134 mg/mL β-glycerophosphate (BGP). The pKa of lactic acid is 3.86; it is not readily ionizable at physiological pH. Likewise, BGP does not contribute significantly to ionic strength: either it is actively crosslinking chitosan or protonated. We therefore estimate that chitosan, commensurate with all hydrogels tested, should have an ionic strength ~120 mM. We also verified experimentally the failure to trap in chitosan was not due to abrogation of PEG affinity for anti-PEG IgG or IgM, as determined by ELISA (Suppl. Fig. 5). Agarose, made in a 1x PBS matrix, has similar ionic strength ~160 mM. The ionic strength of our alginate preparation is also ~120 mM. Thus, a difference in ionic strength of each biogel does not appear to contribute to the differences in their ability to facilitate Ab-mediated trapping.

3.5 Antibody-matrix bonds are likely electrostatic in nature

Charged particles can directly bind biogel matrix constituents due to electrostatic interactions, which can be disrupted by increasing salt concentrations [43]. To determine if antibody-mediated trapping was similarly electrostatic in nature, we added different amounts of sodium chloride to alginate gels (up to 500 mM final concentration) (Fig. 6, Suppl. Fig 2E). The resultant ionic concentration of alginate was therefore ~220 mM- ~1100 mM, well over physiologic ionic strength. 2000 nm COOH beads in alginate remained mostly immobilized in gels with 50 mM additional NaCl, but the fraction of nanoparticles classified as mobile and the $<D_{eff}>$ were increased when 100 and 500 mM NaCl were added (Fig.6 A-B, Suppl. Fig. 2E), Presumably, this was due to the increased sodium interacting with

carboxyl groups on the alginate instead of calcium, destabilizing the gel structure. It prevented the use of higher concentrations of NaCl as well. Introducing an additional 50 mM NaCl also largely eliminated the ability of IgG and IgM to immobilize PEG nanoparticles in alginate, with the $<D_{eff}>$ increased ~50-fold (Suppl. Fig. 2E). Finally, addition of excess salts up to 500 mM NaCl did not substantially alter the affinity of anti-PEG IgG or IgM to PEG (Suppl. Fig. 5). These results suggest that antibody-mediated trapping is due to electrostatic-type interactions.

3.6 Molecular mechanism of antibody-mediated trapping

Previously, Olmsted, et al. [44] found that antibodies are only capable of transient interactions with mucins; the diffusion of IgG and IgM are slowed only ~10-20% and ~50-70%, respectively, in human midcycle cervical mucus compared to their diffusion in PBS. Our group has since shown that the transient and weak nature of antibody-mucin crosslinks is essential for facilitating effective trapping, by allowing antibody to undergo rapid diffusion in mucus and quickly accumulate on the surface of pathogens and nanoparticles, which in turn result in the formation of multiple weakly adhesive crosslinks between the array of Ab bound to the pathogen and the biogel matrix [16]. In other words, Ab must possess some affinity to matrix constituents to mediate trapping in biogels, but the affinity should be inherently weak, non-covalent, and transient. Based on our studies using biogels with an abundance of hydrogen bond (H-bond) donors and acceptors, we present a model in which H-bonds along the polysaccharide backbone of the various biogels investigated here are responsible for the observed IgG- and IgM-matrix interactions necessary to enable trapping of nanoparticles (Fig. 7).

H-bond strength varies widely, ranging from homonuclear sulfur H-bonds with near Van der Waals strength at <1 kcal/mol to homonuclear fluorine H-bonds, which have bond strength equivalent to a weak covalent interaction of 40-45 kcal/mol. H-bonds between groups with a carbon or nitrogen heavy atom typically have strengths of 2-32 kcal/mol, depending on the environment, charge of chemical groups, and resonance [45]. Due to the tight packing of N-glycans within the Fc core [46], we

assume the terminal sugar on each glycan chain to be the most relevant contributor to H-bonding interactions with the biogel matrix, as it is more sterically available for binding. The terminal glycan of mouse IgG_1 is ~20-50% galactose, 10-20% sialic acid, and the rest N-acetyl glucosamine [47]. At physiological pH ~7.4, sialic acid is negatively charged, whereas the remaining glycans are neutral. We thus assumed the key chemical groups distinguishing the different Ab glycans for binding to matrix to be hydroxyl on galactose, N-acetyl groups on sialic acid, and N-acetyl glucosamine terminated glycans. Twenty of 51 N-glycosylation sites on IgM are high-mannose-type, meaning they terminate in mannose glycans, which possess only hydroxyl as a functional group.[41] The other 31 glycosylation sites, however, are 80% occupied and terminate exclusively in sialic acid residues, which possess both carboxylic acid and N-acetyl functional groups.[48] Overall, an average IgM molecule will have ~25-30 glycans terminating in N-acetyl and carboxylic acid groups, compared to a single carboxylic acid appearing of 10-20% of IgG or N-acetyl appearing on 30-70% of IgG.

We first consider the case of agarose, which offers only hydroxyls for binding antibodies. While hydroxyl/hydroxyl H-bonds are possible (e.g., galactose/agarose interactions; Fig. 7A), should this occur, they would be incredibly weak, unassisted by charge according to Gilli's chemical leitmotifs [45]. Hydroxyl/N-acetyl H-bonds (e.g. N-acetyl glucosamine/agarose interactions; Fig. 7B) would be somewhat stronger due to the partial negative charge on the carbonyl making it a better proton receptor; however, since no true charge exists, this is still a relatively weak bond. Hydroxyl/carboxylic acid bonds (e.g. sialic acid/agarose bond; Fig. 7C) would be the strongest of the three. The negative charge of sialic acid at neutral pH allows it to accept a donated proton from an hydroxyl group, creating a negative charge-assisted H-bond that is inherently stronger [45]. However, sialylated IgG represents only a small fraction (10-20%) of the total IgG population. As IgG are usually only singly sialylated, the overall number of strong H-bonds may be too low to facilitate trapping of particles, as reflected by the lack of IgG-mediated trapping observed in agarose gels. In contrast, each IgM has 31 glycan sites that are

80% sialylated (the remaining 20 sites are strictly high-mannose type). Thus, IgM accumulated on the surface of PEG-nanoparticles would possess far greater number of sialic acid/agarose bonds, consistent with the observed IgM-mediated trapping in agarose. While desialylation of IgM does decrease its ability to mediate trapping in agarose, it does not completely abrogate it (Fig. 5); this may be due to the sheer volume of weak hydroxyl/hydroxyl interactions between agarose and the exposed galactose on desialylated IgM.

We next consider alginate, which has a carboxyl group on every monomer along with several hydroxyl groups. At neutral pH, this carboxyl group is negatively charged, capable of accepting donated hydrogen or sharing an environmental proton. Negative charge-assisted H-bonds are classified as strong under the Gilli system [45]. Carboxyl/hydroxyl bonds can form between alginate and all 3 common forms of IgG N-glycans and also are the primary H-bonds present upon desialylation of IgM (Fig. 7G). Carboxyl/N-acetyl H-bonds may also occur (Fig. 7I); due to resonance, amides can readily accept H-bonds on the oxygen atom. An environmental proton, which is common in a near-neutral aqueous environment, can be shared between carboxyl and N-acetyl groups. This may contribute to trapping in alginate gels treated with IgG, as 20-60% of IgG N-glycans are terminated in N-acetyl glucosamine, as well as trapping with IgM, due to the N-acetyl group on sialic acid on Ab N-glycans. Carboxyl/carboxyl H-bonds, which are also possible between alginate and sialic acid (Fig. 7H), similarly require an environmental proton as both molecules are negatively charged at the pH studied (Fig. 7H-I). Relative bond strengths take into account both resonance created as a result of the bond and charges involved. Increased resonance in the carboxyl/N-acetyl bond would result in a slightly stronger bond than that of a hydroxyl/N-acetyl or carboxyl/hydroxyl bond, but both are far weaker than the carboxyl/carboxyl bond or even the oxygen-strengthened carboxyl/N-acetyl bond. However, it is unlikely that carboxyl/carboxyl H-bonds (Fig. 7I) comprise the majority of IgG- or IgM-biogel interactions; while strong, these interactions are not geometrically favored [49]. The sialic acid content on IgG is also low, further limiting

the number of such bonds. Finally, they do not appear necessary, as desialylation of IgM did not appreciably impact its ability to trap nanoparticles in alginate.

Finally, we consider chitosan, whose main functional group of interest is a primary amine. At pH of ~7.5, as used in this study, about 50-100% of these amines would be positively charged. These positively charged nitrogens lead to facile crosslinking with a negatively charged ion, BGP. However, we calculate that >11% of these amine groups should remain available for interaction after crosslinking with BGP. According to Gilli, et al. [45], ionic carboxylic acid groups and ionic amine groups should form a strong H-bond, and therefore amine groups should in theory facilitate trapping, especially in the context of fully sialylated IgM. This was clearly not supported by our experimental findings. We speculate that, given the abundance of hydroxyl groups also present on chitosan, amine/hydroxyl H-bonds (Fig. 7D) may occur between different strands of chitosan, thus outcompeting potentially stronger amine/carboxylic acid H-bonds by sheer volume and proximity (Fig. 7F). This assertion is supported by the fact that IgM facilitates trapping in agarose but not chitosan, implying that the hydroxyl groups on chitosan are not available for IgM (and IgG) to bind. This masking effect does not occur in alginate because carboxylic acid/hydroxyl H-bonds are weaker than amine/hydroxyl H-bonds, and therefore the intramolecular H-bonds are easier to break to be replaced by antibody glycan/biogel H-bonds. Alternatively, ionic carboxylic acid bonds with ionic amine may be too strong, resulting in limited mobility of the antibodies in the chitosan gel and consequently poor trapping potencies.

There may well be a difference in affinity between the anti-PEG IgG and anti-PEG IgM used in this study that might lead to a difference in the number of Ab bound on each particle, which in turn might influence conclusions about trapping potencies of IgM vs. IgG. However, previous studies have demonstrated that at the Ab concentrations used here, both IgG and IgM are sufficient to trap ~85-90% of particles in both basement membrane and laminin.[8] Thus, we do not believe the number of Ab

bound onto the surface of nanoparticles alone could account for why IgM is capable of trapping nanoparticles in select gels that IgG cannot.

4 Conclusion

In this study, we compared the ability of IgG and IgM to mediate trapping of nanoparticles in three biologically relevant hydrogels with distinct functional groups to ascertain the specific moieties with which antibodies may interact in common biogels. In agarose, with polysaccharides possessing only hydroxyl groups, only sialylated IgM mediated trapping. Chitosan, which possesses both hydroxyl and amine groups, facilitated no trapping. Alginate, which is rich in carboxylic acid, meanwhile, facilitated potent nanoparticle immobilization by both IgG and IgM. Based on studies using deglycosylated IgG and desialylated IgM, we propose that antibody-mediated trapping is dominated by hydrogen bonding to carboxyl groups on biogel matrices. When designing biogels with the intent of harnessing Ab secreted by the immune system or provided exogenously to reinforce its barrier properties, evidence presented in this study supports the incorporation of COOH groups in the matrix constituents for efficient immobilization of pathogens.

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6 Disclosures

Mucommune, LLC and Inhalon Biopharma, Inc both seek to harness antibody-mucin interactions to improve protection against or treatment of infections at mucosal surfaces, and have licensed

intellectual property from the University of North Carolina - Chapel Hill (UNC-CH). SKL is a founder of both Mucommune and Inhalon, and owns company stock. SKL's relationship with Mucommune and with Inhalon is subject to certain restrictions under University policy. The terms of this arrangement are being managed by UNC-CH in accordance with its conflict of interest policies.

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