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Nanogel receptors for high isoelectric point protein detection: Influence of electrostatic and covalent polymer-protein interactions

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An aldehyde acrylate-based functional monomer was incorporated into poly(N-isopropylacrylamide-co-methacrylic acid) nanogels for use as protein receptors. The aldehyde component forms dynamic imines with surface exposed lysine residues, while carboxylic acid/carboxylate moieties form electrostatic interactions with high isoelectric point proteins. Together, these interactions effect protein adsorption and recognition.

Protein recognition using synthetic, crosslinked nano- and microgels has been an area of great interest over the last two decades due to their ability to deliver and sequester biologics, specifically for biosensing and drug delivery applications.[1–4] Poly(N-isopropylacrylamide) (P(NIPAM)) represents one of the more studied materials utilized for protein recognition due to its inherent thermoresponsivity. P(NIPAM)-based hydrogels exhibit a lower critical solution temperature (LCST), at approximately 31°C,[5] making these materials widely attractive for a range of biomedical applications. In the field of biosensing, P(NIPAM) hydrogels have been utilized to capture and concentrate protein biomarkers[6–8] due to the semi-selective protein recognition of the material.[9,10]

P(NIPAM) hydrogels are commonly synthesized by free radical precipitation polymerization, due to the robust nature of network formation that is amenable to variety of functional groups. Anionic and cationic monomers are extensively used to promote interactions with proteins or various analytes utilizing electrostatic interactions with charged residues on the protein surface. However, it is well known that crosslinked polymers with ionizable functionalities exhibit a swelling behavior that depends on synthesis parameters, such as the monomer feed ratios, the pK_a value(s) of the monomer(s), the extent of crosslinking, and other factors including the pH and ionic strength of the solution.[3,11,12] For this reason, diffusion of solutes through the polymer network for molecular recognition is impacted by the equilibrium swelling ratio of the hydrogel. Thus, in order to rationally choose monomers that promote favorable interactions with proteins based on their properties (i.e., isoelectric point, amino acid content, etc.), protein biomarkers of interest must first be identified.

Dry eye is highly prevalent in the global population due to its association with many diseases, side-effects of medications, among other factors.[13,14] Despite the high incidence of dry eye, it is frequently underrecognized and can greatly impact a patient's vision and quality of life.[15] Dry eye is currently diagnosed based on several tests which evaluate the ocular surface and tear film production.[13–15] While these tests are standard and necessary, many clinicians and researchers have reported the need for earlier stage identification, validation, and detection of biomarkers from tears to assist in reaching conclusive diagnoses, especially if dry eye is associated with disease onset (e.g., in the autoimmune disease Sjögren's syndrome).

Many proteins are present in tears at varying concentrations, with lysozyme, lactoferrin, lipocalin-1 and IgA being the most abundant.[16,17] Lysozyme and lactoferrin are proteins which exhibit high isoelectric points (pI), while lipocalin-1 and IgA are low pI proteins (see ESI,† Table S1). The difference in isoelectric points of the aforementioned proteins, in addition to their high abundance in tears, makes these analytes strong candidates to target in the development of protein receptors for use in biosensing applications.[9]

One method to impart unique molecular recognition properties in hydrogels is to incorporate dynamic covalent bonding functionalities.[18–20] Covalent bonds are stronger than

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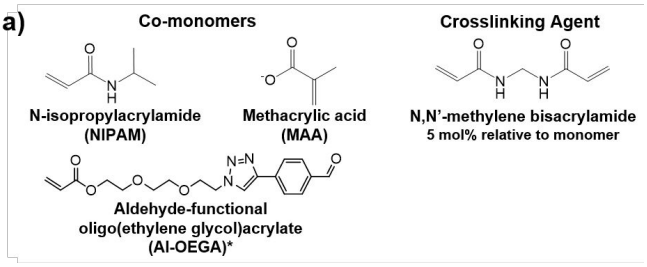
† Electronic Supplementary Information (ESI) available: Full details of material synthesis and characterization are included, as well as additional binding results for all nanogel formulations. See DOI: 10.1039/x0xx00000x

a)



b)

Formulation[a]	NIPAM (mM)	MAA (mM)	Al-OEGA (mM)	Notation[a]
P(NIPAM)	16.5	-	-	NN
P(NIPAM-co-Al-OEGA)	12	-	4.5	NA
P(NIPAM-co-MAA)	12	4.5	-	NM
P(NIPAM-co-MAA_2-co-Al-$OEGA_1$)	12	3.0	1.5	NM_2A_1
P(NIPAM-co-MAA_1-co-Al-$OEGA_2$)	12	1.5	3.0	NM_1A_2

Fig. 1 Monomers (a) and their ratios (b) used in the synthesis of P(NIPAM)-based nanogels.*Al-OEGA was custom prepared by the Anslyn group. All other monomers were purchased commercially. [a]Subscripts refer to the ratio of MAA:Al-OEGA included into the polymerization. Details of monomer and nanogel syntheses are included in the ESI†.

non-covalent interactions, and can be incorporated into neutrally-charged materials alone or in addition to substrates possessing non-covalent interactions (i.e., electrostatic) to achieve high affinity receptors.[21,22]

Anslyn,[23–26] and others,[22,27,28] have previously synthesized novel molecules capable of forming orthogonal, reversible covalent interactions with either diols, hydrazides, or thiols. Furthermore, analysis of the solvent accessible surface area[29] of the identified tear proteins revealed differences in the number of surface exposed lysines (amines) present in both high and low pI proteins (see ESI,† Table S1).

For this reason, nanogels were synthesized based on precipitation polymerization methods previously established in the Peppas lab to formulate P(NIPAM-co-methacrylic acid) (NIPAM-co- MAA) nanogels.[10] This polymerization scheme was adapted to include an aldehyde-functional oligo(ethylene glycol)acrylate (Al-OEGA) monomer (Fig. 1a) capable of forming dynamic hemiaminals and imines with select tear proteins to create a library of nanogels with various molecular recognition properties.

The precipitation polymerization technique uses a surfactant-free synthesis approach to obtain uniform particle size and eliminates potential surfactant-mediated interactions during subsequent protein binding experiments. In addition, this synthesis method has broad applications (e.g., in biosensing and drug delivery) and is advantageous for several reasons: (i) nanogels are synthesized reproducibly, (ii) nanogels are swollen at room temperature, and (iii) monomers capable of forming ionic and covalent interactions with select proteins are easily incorporated. Specifically, MAA was incorporated into the polymerization to promote electrostatic interactions with high pI proteins, while Al-OEGA was incorporated to form covalent bonds with proteins containing surface exposed lysines (Fig. 1).

Nanogels of varying formulations (Fig. 1b) were synthesized via precipitation polymerization in water (experimental details regarding Al-OEGA and nanogel syntheses can be found in the ESI†). Confirmation of nanogel synthesis was obtained by Fourier-transform infrared spectroscopy (see ESI,† Fig. S5). To confirm MAA incorporation, potentiometric titrations of the nanogel suspensions were performed. The results obtained indicated no acid content in NN and NA formulations, and increasing acid content (from least acid to most acid) in NM_1A_2, NM_2A_1, and NM formulations (see ESI,† Fig. S6). In addition, zeta potential was used as an indirect method to confirm MAA incorporation into the synthesized nanogels. Measurements were obtained in 0.1X phosphate buffered saline (PBS, pH 7.4), which was used for subsequent protein binding experiments. The results obtained corresponded to decreased zeta potentials for NM_1A_2, NM_2A_1, and NM nanogels compared to NN and NA nanogels which contain no MAA (see ESI,† Table S2).

When using nanogels as protein receptors, it is important to consider how hydrogels composed of different monomer compositions effect their swelling behavior, which can affect protein diffusion throughout the polymer network. The synthesized nanogels were therefore characterized by transmission electron microscopy (see ESI,† Fig. S7) and dynamic light scattering (see ESI,† Table S4). The hydrodynamic diameters of the synthesized nanogels obtained were largest when increasing concentrations of MAA was incorporated into the polymerization (NM_2A_1 and NM), and smallest when increasing concentrations of Al-OEGA was incorporated into the polymerization (NM_1A_2 and NA).

Once nanogels were characterized, the functionality of the aldehydes to form covalent bonds with lysine residues on the protein surface was evaluated. A standard peptide assay was used to determine adsorption capacity of a custom prepared acyl hydrazide containing peptide[30] to the synthesized nanogels (see ESI,† Fig. S8). The adsorption capacity (Q) was calculated using **Equation 1**, where C_0 and C_e are the initial and equilibrium protein concentrations, respectively, V is the solution volume, and m is the mass of the nanogels.

$$Q = (C_0 - C_e)V/m \quad (\textbf{Equation 1})$$

All Al-OEGA containing nanogels (NA, NM_1A_2, and NM_2A_1) resulted in statistically significant ($p < 0.05$) increases in adsorption capacity compared to results obtained in the formulation without Al-OEGA (NM nanogels, Fig. 2). However, no differences in adsorption capacity were observed between

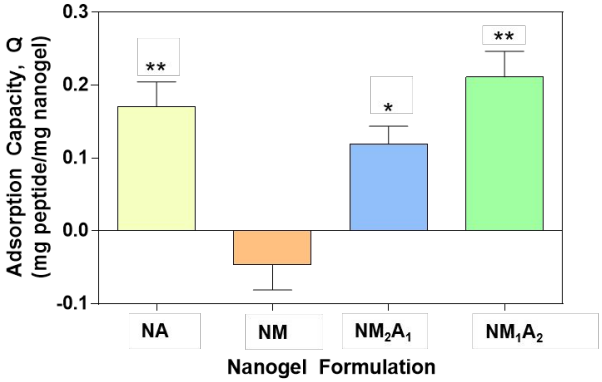


Fig. 2 Characterization of peptide binding to synthesized nanogels. NA: P(NIPAM-co-Al-OEGA); NM: P(NIPAM-co-MAA); NM_2A_1: P(NIPAM-co-MAA_2-co-Al-$OEGA_1$); NM_1A_2: P(NIPAM-co-MAA_1-co-Al-$OEGA_2$). Subscripts in nanogel formulations refer to the ratio of MAA:Al-OEGA included in the polymerization. Data are reported as mean ± standard error of the mean and were analyzed using multiple one-way ANOVAs. * indicates $p < 0.05$, ** indicates $p < 0.01$ compared to NM nanogels.

Protein	Isoelectric Point	Molecular Weight (kDa)	# Solvent Accessible Lysine[a]
Lysozyme	11.3	14.3	5
Lactoferrin	8.7	80	46
IgG	6.5-9.5	153	varies
β-Lactoglobulin	5.2	18	15
Albumin	4.7	66.5	58

Table 1. Properties of proteins used in binding studies.

[a] solvent accessible surface area (SASA) was quantified using the PDBePISA (Protein Data Bank in Europe, Proteins, Interfaces, Structures and Assemblies) SASA tool available online through the European Molecular Biology Laboratory.[29]

Al-OEGA containing nanogel formulations (Fig. 2). These results indicate that the formulation containing the smallest amount of Al-OEGA (NM_2A_1) is sufficient to significantly increase peptide detection (based on adsorption capacity). In addition, increasing Al-OEGA concentration in the nanogel formulations does not directly translate to increasing peptide detection.

To investigate the ability of the synthesized nanogels to serve as tear protein receptors, protein binding studies were performed with select human tear proteins, specifically: lysozyme, lactoferrin, IgG, and albumin (Table 1) in non-competitive environments (buffer alone). Bovine β-lactoglobulin was used as a model protein in place of lipocalin-1 due to its accessibility and similarity in pI, molecular weight, and number of solvent accessible lysines (Table 1). The proteins tested are further classified as high pI proteins (namely, lysozyme, lactoferrin, and IgG), or low pI proteins (β-lactoglobulin and albumin). Upon protein binding at a range of concentrations in 0.1X PBS to NN (see ESI,† Fig. S9) and NA (Fig. 3a), nanogels showed small increases in adsorption capacity as the equilibrium protein concentration increased.

The exception to this was observed for lactoferrin upon binding to NA (Fig. 3a). Lactoferrin is a high pI protein with 46 solvent-accessible lysine residues capable of forming dynamic covalent bonds (DCBs) with Al-OEGA functionalized nanogels. NA nanogels contained the largest molar concentration of Al-OEGA (of the nanogels synthesized), resulting in the increase in adsorption capacity.

When proteins were incubated with nanogel formulations containing MAA (specifically, NM, NM_2A_1, and NM_1A_2), increases in adsorption capacity were observed for high pI proteins (Fig. 3b-d), but no difference in adsorption capacity was observed for low pI proteins (Fig. 3b-d). This protein-polymer recognition is attributed to electrostatic interactions between the negatively charged nanogels and positively charged proteins at pH 7.4. However, as MAA incorporation in nanogels decreased and Al-OEGA incorporation increased (i.e., NM_1A_2), larger differences in adsorption capacity were observed for high pI proteins, suggesting that protein binding is affected by both electrostatic and DCB interactions between proteins and nanogels (Fig. 3d). Despite albumin containing the highest number of solvent accessible lysines of the proteins investigated, there was no observable protein binding (based on adsorption capacity) when utilizing any reported nanogel formulations. We attribute this to charge-charge repulsion between the negatively charged nanogels and negatively charged proteins at pH 7.4 (Fig. 3a-d). This was also observed for NA nanogels, which exhibited a slightly negative zeta potential, (see ESI,† Table S4) likely due to the persulfate groups present in the ammonium persulfate initiator (see ESI†).

Moreover, adsorption capacities of proteins at 0.75 mg/mL for all nanogel formulations containing MAA resulted in statistically significant ($p < 0.001$) increases in adsorption capacities for high pI proteins (lysozyme, lactoferrin, and IgG) compared to NN (see ESI,† Fig. S10) and NA (Fig. 4) nanogels synthesized without MAA. In addition, differences in adsorption capacities for low pI proteins (β-lactoglobulin and albumin) to all nanogels tested were not significant (Fig. 4). The largest adsorption capacity obtained was observed for lactoferrin to NM and NM_1A_2 nanogels due to its positive charge and increased number of solvent accessible lysines. However, while incorporation of Al-OEGA into the nanogels increased separation of the protein binding events (based on adsorption capacity) at various high pI protein concentrations (Fig. 3d), our results demonstrate that protein binding to nanogels is still primarily governed by electrostatic interactions. Nevertheless, the impact of Al-OEGA inclusion into the nanogels resulted in increased adsorption capacity of lactoferrin (a high pI protein with increased solvent accessible lysines) and was more apparent at low equilibrium protein concentrations (Fig. 3d). This variation may translate to increased protein detection sensitivity.

In summary, a library of P(NIPAM) nanogels composed of monomers capable of forming both non-covalent and dynamic covalent interactions with select tear proteins was synthesized. The identity of the ionizable and covalent groups within the nanogels impacted their swelling behavior, charge character, and, consequently, the protein binding behavior (based on adsorption capacity). The protein binding studies indicate that electrostatic interactions are the main driving force behind

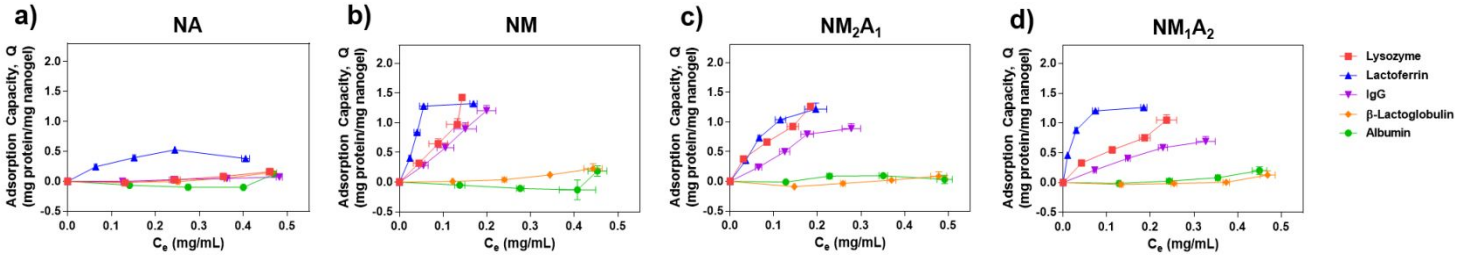


Fig. 3 Characterization of adsorption capacities for select proteins to synthesized nanogels as a function of equilibrium protein (Ce) concentration. Adsorption capacities were determined from equilibrium protein binding experiments performed with a range of initial protein concentrations (0-1 mg/mL) in 0.1X PBS. a) Adsorption capacities of proteins to P(NIPAM-co-Al-OEGA) nanogels (NA). b) Adsorption capacities of proteins to P(NIPAM-co-MAA) nanogels (NM). c) Adsorption capacities of proteins to P(NIPAM-co-MAA_2-co-Al-$OEGA_1$) nanogels (NM_2A_1). d) Adsorption capacities of proteins to P(NIPAM-co-MAA_1-co-Al-$OEGA_2$) nanogels (NM_1A_2). Subscripts in nanogel formulations refer to the ratio of MAA:Al-OEGA included in the polymerization. Data are reported as mean ± standard deviation.

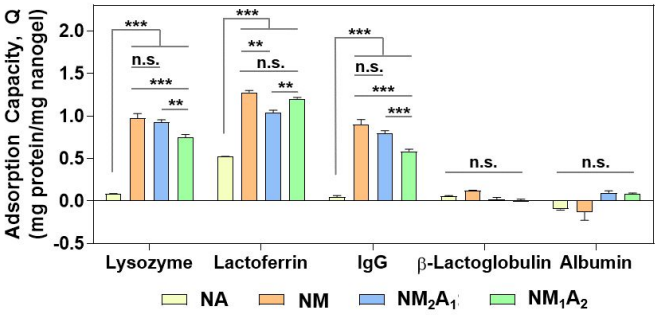


Fig. 4 Adsorption capacities of select proteins to synthesized nanogels. Adsorption capacities were determined from equilibrium protein binding experiments with initial protein concentrations of 0.75 mg/mL in 0.1X PBS. NA: P(NIPAM-co-Al-OEGA); NM: P(NIPAM-co-MAA); NM_2A_1: P(NIPAM-co-MAA_2-co-Al-$OEGA_1$); NM_1A_2: P(NIPAM-co-MAA_1-co-Al-$OEGA_2$). Subscripts in nanogel formulations refer to the ratio of MAA:Al-OEGA included in the polymerization. Data are reported as mean ± standard error of the mean and were analysed using multiple two-way ANOVAs. ** indicates $p < 0.01$, *** indicates $p < 0.001$, n.s. indicates comparison is not significant.

protein-polymer interactions and are in agreement with findings from previous literature reports.[2,4,10] Incorporation of monomers which form covalent bonds with select amino acid residues on the protein surface does have a positive impact on adsorption capacity, but ionic interactions remain the predominant cause for protein binding throughout the bulk of the nanogels.

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Conflicts of interest

There are no conflicts to declare.

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