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Enzyme Activity Inhibition of α-Amylase using Molecularly Imprinted Polymer (MIP) Hydrogel Microparticles.

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Molecularly imprinted polymer, hydrogel, enzyme inhibition, alpha-amylase

Abstract

Molecularly imprinted polymers (MIPs) are a class of synthetic recognition materials that offering a cost-effective and robust alternative to antibodies. While MIPs have found their predominant use in biosensing and diagnostic applications, their potential for alternative uses, such as enzyme inhibition, remains unexplored. In this work we synthesised a range of acrylamide-based hydrogel MIP microparticles (35 µm) specific for the recognition of α-amylase. These MIPs also showed good selectivity towards the target protein with over 96% binding of the target protein, compared with the control non-imprinted polymer (NIP) counterparts. Specificity of the MIPs was determined with the binding of a non-target proteins, Trypsin, Human Serum Albumin (HSA) and Bovine Serum Albumin (BSA). The MIPs were further evaluated for their ability to inhibit α-amylase enzymatic activity, showing a significant decrease in activity. These findings highlight the potential of MIPs as enzyme inhibitors, suggesting an innovative application beyond their conventional use.

Introduction

Type 2 diabetes is a form of diabetes that is characterised by high blood sugar, insulin resistance and relative lack of insulin. Symptoms include increased thirst, fatigue, frequent urination and unexplained weight loss, and if left untreated can lead to heart disease, stroke, blindness, kidney failure and poor blood flow resulting in limb amputation. Obesity and lack of exercise is the predominate cause of type 2 diabetes and is about 90% of all diabetes cases.[1,2] Treatment for the condition requires the lowering

of blood sugar levels, with initial treatment being exercise and dietary change. Failing that medication is then required.[3]

Amylase is a digestive enzyme that catalyses the hydrolysis of starch, a polymeric carbohydrate, into sugars.[4] There are several variations of amylase, with the predominate form being α-amylase which is found in humans and mammals, as well as being found in seeds and fungi.[5] In humans, α-amylase is predominantly found in pancreatic juices and saliva, where it breaks down starch into the sugars maltose and dextrin.[4] Excess amounts of these sugars can lead to significant issues, like hyperglycemia, causing problems in people that suffer from Type 2 diabetes.[3,5] Especially, as carbohydrate digestibility has been related to elevated postprandial blood glucose. One approach to potentially reduce postprandial hyperglycemia is to limit the activity of carbohydrate digestive enzymes, particularly within the digestive tract. Thus, the strict controlling of postprandial blood glucose through inhibition of α-amylase, could be significant for the prevention and treatment of diabetes. The use of α-amylase inhibitors has shown to act as carbohydrate blockers, thus limiting the digestibility and absorption of carbohydrate in the gastrointestinal diet.[6]

Small molecules such as phenolic acids, tannins, anthocyanins, and flavonoids are reported to be potent inhibitors of α-amylase. Their digestibility significantly influences their enzymatic inhibition, thus not all small molecule inhibitors are readily available.[7,8] Additionally, some small molecules have also shown inhibition for other enzymes, such as trypsin. The inhibition of non-target enzymes can cause significant issues leading to serve consequences and side-effects.[7,8] Peptides and the protein α-amylase inhibitor have also been shown to be good inhibitors of α-amylase, offering the target specificity, that the small molecules lack.[9] While being costly to produce, they also lack robustness and are susceptible to degradation and denaturation, especially in the low pHs found in stomach acid. Molecularly imprinted polymers (MIPs) are synthetic recognition materials that have the potential to act as α-amylase inhibitors due to their stability, affinity and specificity.

Molecularly imprinted polymers (MIPs) are a class of synthetic recognition materials that offer the potential for rational design and selectivity of any given target. Typically produced using a self-assembly protocol, functional monomers are arranged around a template (target molecule) through electrostatic forces. The monomers are then crosslinked together, encapsulating the target inside the polymer matrix.[10,11] Post-polymerisation processing and subsequent removal of template, results in cavities within the polymer that are specific in shape, size and functionality to the template. It is within these cavities, where selective binding of targets takes place.[12] Studies have shown that these synthetic recognition materials are able to achieve excellent recognition for a variety of small molecular weight targets as well as protein biomarkers.[13,14] These materials have been produced in all shapes and size, from bulk microparticles, to thin-films and nanoparticles (nanoMIPs), with affinities (K_D values) in the

micromolar to nanomolar range, consistent with that of poly and monoclonal antibodies.[15–17] This high specificity has made them an excellent choice as a viable alternative to antibodies, particularly in diagnostic and biosensing applications.

MIPs have shown considerable success and as viable alternatives to antibodies, particularly as they match recognition, whilst also offering a robustness to the extremes of pH and temperature, that would typically denature an antibody making it ineffective.[18] This strength has led them to be used in a range of applications and finding success in the role as the recognition materials in biosensor/diagnostic applications. It is in the field of sensing, where MIPs have truly found their niche, producing electrochemical, optical, gravimetric, and ELISA type sensors with sub nanomolar limits of detection (LOD).[19–21] For all the success that MIPs have had, there appears to be limited research outside the realm of biosensors. The have been a few studies that show MIPs as potential drug delivery systems,[22–25] but as off yet, limited work on inhibition of enzymatic activity. The work of Xu et al. used molecularly imprinted technology to produce MIPs that were capable of selectively binding trypsin leading to an inhibition of trypsin activity and offering potential therapeutic applications.[26] While Piletsky et al. used MIP nanoparticles (nanoMIPs) to target intracellular epitopes, enabling the modulation of epidermal growth factor receptor (EGFR) activity.[27] This highlights the possibility for using MIPs in a wide variety of applications, other than sensing.

In this work, we have developed and produced a series of molecularly imprinted hydrogel MIPs for the specific recognition and selective binding of α-amylase. The MIPs were produced in a micron sized particle, as this allows for ease of production, while also rapidly producing large number of particles. Using an acrylamide-based monomers, allowed for the inclusion of a variety of functional groups, exploring their effects on recognition.[28] Additionally, being water soluble allows for polymerisation to occur without the need for organic solvents, allowing the use of protein molecules as templates in the molecularly imprinting process and prevents potential protein denaturation that could occur with organic solvent use. The best performing MIPs were then investigated for their use as potential enzyme inhibitors, offering a new possibility of inhibitor materials.

Methodology

Materials

α-Amylase, Acrylamide (AAm), Ammonium Persulfate (APS), Bovine Serum Albumin (BSA), glacial acetic acid (AcOH), Human Serum Albumin (HSA), *N*-(3-aminopropyl)methacrylamide hydrochloride

(NAPAm) *N*-(hydroxymethyl)acrylamide (NHMAm), *N*-isopropylamide (NiPAm), *N,N'*-methylenebis(acrylamide) (mBAm), sodium dodecyl sulfate (SDS), and tetramethylethyldiamide (TEMED), trypsin were all purchased and used without purification from Sigma-Aldrich, Poole, Dorset, UK.

Methods

Solution Preparation.

A solution of 10% (w/v):10% (v/V) SDS:AcOH was prepared for use in the protein elution (template removal stages), before target/non-target reloading stages. SDS (10 g) and AcOH (10 mL) were dissolved in 90 mL of MilliQ water, to produce 100 mL of the elution solution.

MIP Preparation.

MIP hydrogel microparticles were produced, using an existing methodology [14], whereby a hydrogel is produced using a 10% crosslinking ratio of monomer and crosslinker was found to be optimum for protein binding, in terms of efficiency and specificity. Four different MIPs were produced using a range of different functional monomers (AAm, NHMAm, NiPAm, NAPAm), and a crosslinking density of 10%, specific for α-amylase as a template, using the following protocol.

Into an Eppendorf tube, α-amylase (12 mg) was dissolved in 970 µL of MilliQ water then vortexed for 1 minute. Next the functional monomer (0.76 mmol) was added, vortexed for 1 minute and allowed to self-assemble around the template for a further 1 minute. Next, the crosslinker (mBAm) was added at a ratio of 9:1 by weight, and the mixture was vortexed for a further minute, before the addition of 10 µL 5% TEMED (v/v) solution and 20 µL 5% APS (w/v) solution. This mixture was vortexed for 1 minute. The solutions were then purged with nitrogen for 5 minutes and polymerisation occurred overnight at room temperature (approximately 20 °C). Corresponding non-imprinted polymers (NIPs) were produced using the same protocol, but in the absence of the protein template, as a comparative control.

After polymerisation, the gel monoliths, were granulated separately using a 35-micron sieve. The gels were then washed with two 1 mL volumes of the 10% (w/v):10% (v/v) SDS:AcOH solution, allowing for the denaturation and subsequent removal of the template protein from the MIP cavities. The gels were then washed with 1 mL volumes of MilliQ water, until all the SDS:AcOH solution was removed, approximately five washes. Each wash step consisted of addition of SDS:AcOH solution/MilliQ water followed by vortex for 1 min, the centrifuged for 10 mins. It should be noted that at this stage it is empirical for the SDS:AcOH solution is completely removed from the washed MIP. Failure to do so could result in the prevention of any target protein being able to rebind in the future, having a negative

effect of the specific recognition properties of the MIP. The corresponding NIPs were also processed in the same manner.

MIP Rebinding Studies.

The binding properties of the conditioned MIPs and corresponding NIPs were investigated and characterised using Nanodrop UV/Visible spectrometer. Each hydrogel (20 mg) was treated with 60 µL of amylase (target protein) solution between the concentration ranges (0.4-3.0 mg mL^{-1}). After the protein solutions were added to the MIP/NIP, the polymer/protein solutions were vortexed for 1 minute and allowed to associate at room temperature (approximately 20 °C) for 5 minutes. Following this, the hydrogels were then centrifuged for 5 minutes, and the supernatant was collected and analysed by UV/Vis spectroscopy (at 210 nm). Selectivity of the MIPs/NIPs was investigated through the non-specific binding of a non-target proteins (Trypsin, HSA, and BSA), with supernatant subsequently, analysed at 276 nm.

The amount of the target (or non-target) protein, bound to the polymers *B*, was calculated by the subtraction of the concentration of the free protein, [FreeProtein], from the initial protein concentration, and determined as a mean average of three measurements. Scatchard analysis was performed using the average results from the binding studies of the MIPs with 60 µL of known concentration (0.4-3) mg mL^{-1}) of the target and non-target proteins, with analysis undertaken using the Scatchard equation (Equation 1).

$$\frac{B}{[FreeProtein]} = (B_{max} - B)K_a \qquad (1)$$

Where, K_a is the association constant and $B_{\max}$ is the theoretical estimate of the maximum number of binding sites.

Thus, production of Scatchard plots (bound concentration/unbound concentration versus bound concentration) allows for determination of association constants (K_a) form the gradient of the slope of the line along with the theoretical maximum number of binding sites (B_{max}) from the intercept of the slope.

Amylase Inhibition Assay.

Phadebas® Amylase Test crushed powder (20 mg) was dissolved in 400 µL of water, next 50 µL of α-amylase solution (2 mg mL^{-1}), premixed with 20 mg of MIP/NIP was added and the solution was vortexed. The mixture was placed in a water bath at 37 °C to initiate the reaction. After 15 minutes

500 µL of 1 M sodium hydroxide solution was added to the solution and vortexed to terminate the reaction. The mixture was then centrifuged for 5 minutes, with the supernatant analysed using a using Nanodrop UV/Visible spectrometer at 620 nm. An α-amylase control (without and MIP or NIP) was also completed using the same protocol, but without premixing MIP and NIP, with the α-amylase. This allowed the determination of free α-amylase activity.

Results and Discussion

A series of MIP hydrogel microparticles were successfully synthesised, using either AAm, NHMAm, NiPAm, or NAPAm, as the functional monomer, mBAm as the crosslinker, and water as the polymerisation solvent. The use of organic solvents was traditionally used in the production of MIPs, but these readily denature proteins, making them unsuitable for the imprinting of Proteins.[10] Acrylamide-based monomers can offer a wide-range of functionality, whilst also being water-soluble.[14] These are important factors when considering the molecular imprinting of proteins, due to the fragility and stability of proteins.[29] With this regard, AAm and NHMAm were specially chosen for their ability for producing high performing hydrogel-based MIP microparticles for protein target.[29] While NiPAm and NAPAm were investigated got their use in high performing MIP nanoparticles but have yet to be investigated with hydrogel-based MIP microparticles.[30]

The hydrogels were initially produced as a 1 g polymer monolith, which after grinding and sieving through 35 µm sieve, produced microparticles with an approximate yield of 60% (0.6 g of microparticles for use). The acrylamide-based functional monomers were chosen due to their water solubility along with known high performance for producing recognition materials for protein targets. Removal of the target (α-amylase) protein, was achieved using a well-established protocol of a series of SDS:AcOH, washes. This solution efficiently removes of proteins from hydrogel-based MIPs by denaturing the protein and prevent the electrostatic forces that complex the α-amylase to the MIP.[31] Subsequent washes then completely remove α-amylase from the MIP leaving a MIP particles with free specific cavities, capable on rebinding the target (α-amylase) protein.[31]

FTIR-ATR analysis of the hydrogel MIPs is shown in Figures S1-S4. The FTIR spectra produced is characteristic of polyacrylamide-based hydrogels, particularly those with a high-water content. The spectra (Figure S1-S4) exhibit strong broad peaks with the range of 3259-3381 cm^{-1}, which is assigned to the O-H stretching of water. The peak displayed here is particularly large and broad and is to be expected because of the high-water content of the hydrogel. The peaks displayed in the 2923-2989 cm^{-1} region are due to the C-H stretching peaks from the polymer. The strong and broad peaks that are displayed in the range of 1629-1658 cm^{-1} can be assigned to the C=O stretching within the amide functionality within the polymer hydrogels and again are broader than would be traditionally seen, due potential hydrogen bonding effects with water molecules. While the weak and sharp peaks in the same area 1535-1610 cm^{-1} are due to N-H bending within the amide functionality. The broad and weak peaks at 974-1040 cm^{-1} can assigned to the C-N stretching peak and again are broader than expected and again is potentially due to hydrogen bonding effects, caused by the large volume of water

molecules within the hydrogel. Peak broadening occurs when there are large concentrations of molecules within a sample are analysed, whereby each molecule can hydrogen bond to a slightly different extent.[32] The infrared absorptions thus occur at differing frequencies for each of these bonds, resulting in a broadening infrared peak as an average of all these slightly different absorptions.[33,34] This highlights that the strong interactions between water and functional groups within hydrogels can significantly impact peak wavenumbers and shape.[28] Figure S4 shows and additional peaks at 2854 cm^{-1} and 1261 cm^{-1} that are not displayed in the other FTIR spectra, so can only be due to the N-H and C-N (respectively) stretching of the amine salt within the NAPAm monomer.

Rebinding Experiments

The reloading of the target protein (α-amylase) on to the MIP microparticles is presented in Table 1 and Figure 1 and shows that the NHMAm MIP produces the best recognition for α-amylase with 96.2% rebinding of the target protein. While the NAPAm MIP was the worst performing MIP with a 79.3% rebind (Table 1). In terms of MIP rebinding performance and efficiency, the monomers investigated can be order as: NHMAm > AAm > NiPAm > NAPAm, with the high performance of NHMAm to be expected. This ordering MIP rebinding performance and efficiency is expected to be due to interactions within the monomer-template complex, prepolymerisation. It is expected that the better performing MIPs is due to stronger interactions between the monomer and template, which leads to cavities within the polymer matrix that can offer stronger rebinding opportunities. This is exemplified computational modelling shows that NHMAm is an excellent choice for protein targeting hydrogel-based MIPs, due to more negative ΔG values (kcal mol^{-1}) across a range of potential bindings sites across the surface of a protein.[14] This results in a strong monomer-template prepolymerisation complex, with multiple monomer interactions around the target, which subsequently produces a highly selective imprinted cavity within the polymer. While this work investigates monomer-target complexation with the protein myoglobin, it is still relevant within the scope of this work. While the protein, myoglobin, differs in shape, length, sizes and amino acid order, to α-amylase, the surface areas of both proteins are still produced from the same amino acids. This means the monomer-protein interactions will be relatively the same, albeit differing in orientation.

Table 1. Association constant (K_a) values for the different hydrogels MIP microparticles and their corresponding NIPs

	Percentage of Protein Bound (%)

Monomer	MIP (α-amylase)	NIP (α-amylase)	MIP (Trypsin)	NIP (Trypsin)	MIP (HSA)	NIP (HSA)	MIP (BSA)	NIP (BSA)
AAm	**83.2** *(± 0.6)*	32.5 *(± 1.1)*	38.87 *(± 5.5)*	36.1 *(± 5.7)*	40.2 *(± 4.5)*	34.1 *(± 7.5)*	37.4 *(± 1.3)*	35.5 *(± 1.5)*
NHMAm	**96.**2 *(± 3.3)*	37.8 *(± 2.2)*	33.39 *(± 0.5)*	37.3 *(± 4.5)*	36.9 *(± 1.8)*	41.8 *(± 2.2)*	42.3 *(± 2.9)*	33.8 *(± 0.9)*
NiPAm	81.3 *(± 2.1)*	76.7 *(± 2.2)*	76.6 *(± 5.7)*	65.2 *(± 5.2)*	68.0 *(± 8.3)*	73.2 *(± 3.6)*	72.4 *(± 3.7)*	68.6 *(± 3.8)*
NAPAm	79.3 *(± 1.3)*	77.1 *(± 2.8)*	78.5 *(± 6.9)*	70.6 *(± 6.5)*	71.3 *(± 5.2)*	79.9 *(± 9.2)*	63.4 *(± 3.0)*	72.6 *(± 2.8)*

The specific recognition capabilities of hydrogel MIPs for the target protein (α-amylase) were investigated through a series of rebinding experiments. By comparing the binding of the MIPs with that of their corresponding NIP controls Figure 1), an imprinting factor (IF) can be calculated as a ratio of MIP binding versus NIP binding (Equation 2) and is a traditional way of assessing the strength of interactions of the imprinted polymer with the target molecule. In this regard, the higher the IF value, the greater selectivity for the target molecules the MIP is.[35]

$$IF = \frac{\%\, target\ protein\ to\ MIP}{\%\, target\ protein\ to\ NIP} \tag{2}$$

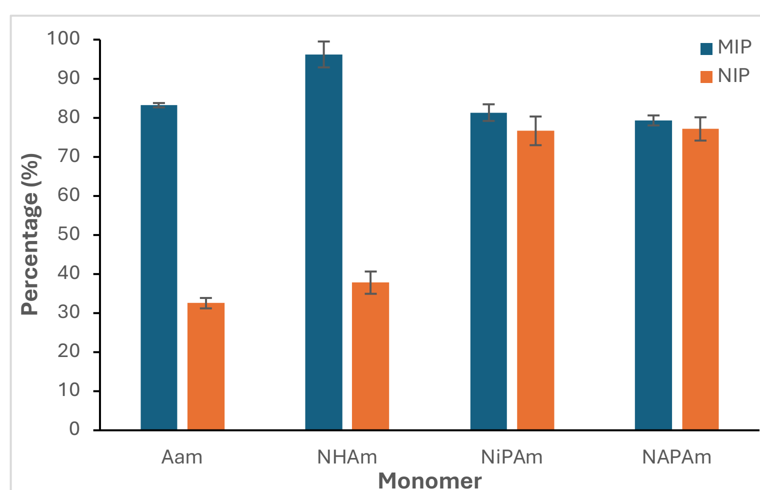

Figure 1. Percentage of α-amylase target protein binding to the four different hydrogel MIPs (blue) and their corresponding NIPs (orange). N=3

Polyacrylamide hydrogels have been used to fix enzymes and maintain their enzymatic ability, with polymers that could be considered as the NIPs used in this study.[36] Whilst, calculating IF values are a

valuable way of assessing MIP performance against its control polymer, studies have shown that there are subtle differences within the polymer matrix between the MIP and the NIP. These differences are due the cavities created during the imprinting process with the work of Kimhi et al. showing the MIP a more porous material, which inherently effects the binding performance of the MIP and NIP materials.[37] As such, comparing the binding performance of the MIP with the target protein versus a non-target protein (Figure 2) to produce a selectivity factor (SF) value (Equation 3) has now become a more favourable assessment of MIP performance, as it truly shows the binding potential of the imprinted cavities.

$$SF = \frac{\%\ target\ protein\ to\ MIP}{\%\ non-target\ protein\ to\ MIP} \quad (3)$$

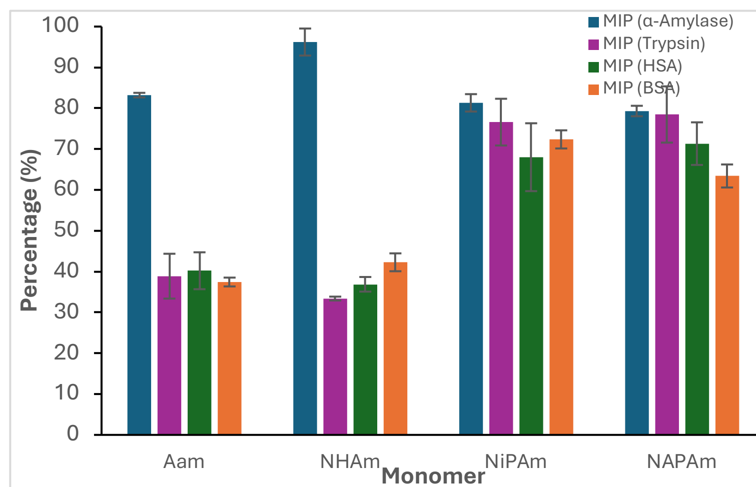

Figure 2. Percentage of α-amylase target protein (blue) and non-target proteins; Trypsin (purple), Human Serum Albumin (HSA) (green) and Bovine Serum Albumin (BSA) (orange) binding to the four different hydrogel MIPs. N=3.

The calculated IF and SF values are presented in Table 2 and shows that NHMAm, AAm MIPs have IF values of 2.55 and 2.56, respectively, and SF values of 2.14 and 2.88 for trypsin, 2.07 and 2.61 for HSA, and 2.28 and 2.22 for BSA, respectively. This shows that these MIPs are highly selective and specific for the binding of the target protein (α-amylase). Contrary to this, the NiPAm, and NAPAm MIPs have IF values of 1.06 and 1.03, respectively, and SF values of 1.06 and 1.01 for trypsin, 1.19 and 1.11 for HSA, and 1.12 and 1.25 for BSA, respectively. These low values highlight the non-selectivity and specificity of nature of the NiPAm and NAPAm, meaning any binding is not due to any MIP effect and is because the polymers produced are capable of effectively binding any non-target protein, mostly likely due to intrinsic charge of the formed gel.

Table 2. Imprinting Factor (IF) values and Selectivity Factor (SF) values for the four different hydrogel MIPs.

Monomer	IF (MIP/NIP)	SF-MIP (α-amylase /Trypsin)	SF-MIP (α-amylase /HSA)	SF-MIP (α-amylase /BSA)
AAm	2.56	2.14	2.07	2.22
NHMAm	2.55	2.88	2.61	2.28
NiPAm	1.06	1.06	1.19	1.12
NAPAm	1.03	1.01	1.11	1.25

Additionally, the SF values of the NIPs were also calculated (Table S2), with values between 0.87 and 1.25 and highlight that the NIPs will bind the target protein (α-amylase) the same as the non-target proteins (Trypsin, HSA and BSA), showing that the control polymer (NIP) is not selective will effectively bind anything. It should be noted, that whilst both α-amylase and BSA are found together in bovine species,[38] BSA is not found in the human body, whereas, trypsin and HSA are, and as an alternative (human version) competitor protein of α-amylase that can also be used to investigate MIP specificity. With BSA being a homolog of HSA, the two proteins have been shown to be structurally similar, sharing a high degree of structural similarity. This has resulted in ligand-binding affinity studies employing BSA as an HSA substitute because of the similar size and environment of binding pockets.[39] Thus, for the purpose of this study, BSA should be also considered as an appropriate additional protein to showcase MIP specificity, and it can be reasonable assume that the MIP produced in this case is specific only for α-amylase.

Batch rebinding experiments were used to investigate the binding behaviour of the MIPs and their corresponding NIPs, with the association constants (K_a values) of the polymers estimated using the Scatchard equation (Equation 1). The Scatchard plots for the MIPs (Figure S5-S8) display a linear transformation, where the slope of the line represents the association constant (K_a), these are presented in Table 3.

Table 3. Association constant (K_a) values for the different hydrogels MIP microparticles.

Monomer	K_a Values (M^{-1})
AAm	5.49×10^{6}
NHMAm	5.86×10^{6}
NiPAm	8.69×10^{5}

NAPAm	9.58×10^5

Table 3 shows that the NHMAm and the AAm are the best performing MIPs with the greatest affinity providing K_a values of 5.86×10^6 and 5.49×10^6. this is an approximately a six-fold improvement over the NiPAm and NAPAm MIPs, which have K_a values of 8.69×10^5 and 9.58×10^5, respectively. This increase in affinity is potentially due to the high performing behaviour of the NHMAm and AAM monomers and their ability to perform strong hydrogen-bond interactions within several potential binding sites around the surface of a protein target molecule, during the prepolymerisation process. This allows for the creation of more selective and specific binding cavities within the MIP, thus better performance. Figure S5-S8, largely display linearity, enough to produce correlation (R^2) values greater than 0.91 and is good enough for affinity (K_a value) estimations. However, slight plateauing can be seen and is to be expected as the MIPs reach their maximum binding capacity.[30] It should be noted that, the K_a values of the corresponding NIPs were unable to be calculate, due to that Scatchard plots not displaying linearity, this further highlights the selective nature of the control polymers.

With this regard, the NHMAm and AAm MIPs (and corresponding NIPs were investigated for their ability to inhibit the enzymatic activity of α-amylase. This was achieved by premixing the MIP (or NIP) with a α-amylase, a Phadebas® Amylase Test, was then used to determine enzymatic activity and is displayed in Table 4.

Table 4. Enzymatic activity of α-amylase determined using Phadebas® Amylase Test, after being premixed with MIP (or NIP). N = 3

Monomer	**Activity (µmol $s^{-1}L^{-1}$)**	
	MIP	**NIP**
AAm	20.4 (± 0.2)	40.3 (± 0.3)
NHMAm	15.4 (0.1)	39.6 (± 0.1)
None[a]	*49.2 (*± 0.1)	

[a] Activity of the α-amylase without the addition of any MIP or NIP

The enzymatic activity of the α-amylase was shown to be 49.2 µmol $s^{-1}L^{-1}$ (Table 4). The introduction of either MIP (AAm or NHMAm) to the α-amylase, shows a significant decrease in enzymatic activity, with 20.4 and 15.4 µmol $s^{-1}L^{-1}$ for AAm and NHMAm MIPs, respectively. This is shows an approximate 58% and 68% decrease in α-amylase activity for AAm and NHMAm MIPs, respectively. The NIP control polymers showed a slight decrease in enzymatic activity, with 40.3 and 39.6 µmol $s^{-1}L^{-1}$ for AAm and

NHMAm. upon addition to the α-amylase. This is due to the slight non-selective binding that was displayed by the NIPs and is still significantly less than the decrease in activity displayed by the MIPs, with only approximately 18% decrease in α-amylase activity, for both NIPs. This highlights further the selectivity of the MIPs and that while they have not shown complete reduction of α-amylase activity, they have shown a significant decrease. As the binding event between the MIP and α-amylase is expected to be at the surface of the enzyme, and produced as an artifact of the self-assembly process, whereby enzyme orientation in prepolymerisation complexation is not controlled. This means there is a potential for some active sites to be exposed, which explains why enzyme activity is not completely reduced to zero. As the MIPs are interacting with α-amylase (temporary or permanent) in a way that reduces the rate of the enzyme-catalysed reaction, this can be considered as enzyme inhibition[40] with further studies involving new functional monomers and MIP particle size needed to increase the α-amylase inhibition even further.

Conclusion

In this study, we successfully prepared a series of highly specific molecularly imprinted hydrogel microparticles (MIPs) targeting the protein α-amylase. Through a straightforward self-assembly process, acrylamide-based MIPs were synthesized, forming a bulk monolith subsequently processed into microparticles. These MIPs demonstrated excellent performance and selectivity, with N-hydroxymethylacrylamide (NHMAm) showing the best overall efficacy. The performance hierarchy for the monomers was NHMAm > acrylamide (AAm) > N-isopropylacrylamide (NiPAm) > N-(3-aminopropyl)methacrylamide hydrochloride (NAPAm).

The MIPs' performance was comparable to other hydrogel-based MIPs for different protein targets, indicating consistency in these materials' behaviour. Scatchard plot analysis revealed enhanced affinity towards the template protein, particularly for NHMAm and AAm, highlighting their strong hydrogen-bonding interactions during the pre-polymerization phase. Specificity and selectivity were further validated using non-imprinted polymers (NIPs) and similarly sized non-target proteins. The calculated Imprinting Factor (IF) and Selectivity Factor (SF) values for the MIPs clearly demonstrated selective recognition.

The best performing NHMAm and AAm MIPs were then used for the inhibition of enzyme activity of α-amylase in the digestion of starch during a Phadebas® Amylase Test, showing a dramatic reduction in enzymatic activity. The development of these unique microparticles offer a simple and effective method to produce a robust biorecognition material in the form of microparticles. The gentle

polymerisation conditions are optimal for protein imprinting and allow for the template to retain its shape during the polymerisation process, preventing target denaturation. Furthermore, the use of these materials as enzyme inhibitors, showcase the potential of these materials to be used in new applications, other than as diagnostic recognition materials. This offers multiple opportunities to use these materials in potential therapeutic applications, of which we are currently exploring opportunities to use these types of materials further

Supporting Information

- Monomer and crosslinker masses (mg) used to produce the MIPs (and corresponding NIPs), at a ratio of 9:1 by weight. FTIR spectra of the MIPs. Scatchard plots analysis of the binding of α-amylase binding to the different MIPs. Selectivity Factors (SF) of the NIPs for the different hydrogels.

Conflicts of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported here.

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Contributions:

CIW: Investigation. CB: Investigation, Supervision. CLH: Investigation. AQS: Funding Acquisition, Writing – Original Draft Preparation. NWT: Funding Acquisition, Writing – Original Draft Preparation, Supervision, Resources. MVS: Conceptualization, Methodology, Formal Analysis, Writing – Original Draft Preparation.

References

(1) Klein, S.; Gastaldelli, A.; Yki-Järvinen, H.; Scherer, P. E. Why Does Obesity Cause Diabetes? *Cell Metab* **2022**, *34* (1), 11–20. https://doi.org/10.1016/j.cmet.2021.12.012.

(2) Chandrasekaran, P.; Weiskirchen, R. The Role of Obesity in Type 2 Diabetes Mellitus—An Overview. *Int J Mol Sci* **2024**, *25* (3), 1882. https://doi.org/10.3390/ijms25031882.

(3) Ch'ng, L. Z.; Barakatun-Nisak, M. Y.; Wan Zukiman, W. Z. H.; Abas, F.; Wahab, N. A. Nutritional Strategies in Managing Postmeal Glucose for Type 2 Diabetes: A Narrative Review. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews* **2019**, *13* (4), 2339–2345. https://doi.org/10.1016/j.dsx.2019.05.026.

(4) Zakowski, J. J.; Bruns, D. E. Biochemistry of Human Alpha Amylase Isoenzymes. *CRC Crit Rev Clin Lab Sci* **1985**, *21* (4), 283–322. https://doi.org/10.3109/10408368509165786.

(5) Azzopardi, E.; Lloyd, C.; Teixeira, S. R.; Conlan, R. S.; Whitaker, I. S. Clinical Applications of Amylase: Novel Perspectives. *Surgery* **2016**, *160* (1), 26–37. https://doi.org/10.1016/j.surg.2016.01.005.

(6) Gong, L.; Feng, D.; Wang, T.; Ren, Y.; Liu, Y.; Wang, J. Inhibitors of A-amylase and A-glucosidase: Potential Linkage for Whole Cereal Foods on Prevention of Hyperglycemia. *Food Sci Nutr* **2020**, *8* (12), 6320–6337. https://doi.org/10.1002/fsn3.1987.

(7) Di Stefano, E.; Oliviero, T.; Udenigwe, C. C. Functional Significance and Structure–Activity Relationship of Food-Derived α-Glucosidase Inhibitors. *Curr Opin Food Sci* **2018**, *20*, 7–12. https://doi.org/10.1016/j.cofs.2018.02.008.

(8) Horii, S.; Fukase, H.; Matsuo, T.; Kameda, Y.; Asano, N.; Matsui, K. Synthesis and .Alpha.-D-Glucosidase Inhibitory Activity of N-Substituted Valiolamine Derivatives as Potential Oral Antidiabetic Agents. *J Med Chem* **1986**, *29* (6), 1038–1046. https://doi.org/10.1021/jm00156a023.

(9) Uraipong, C.; Zhao, J. Rice Bran Protein Hydrolysates Exhibit Strong in Vitro A-amylase, B-glucosidase and ACE-inhibition Activities. *J Sci Food Agric* **2016**, *96* (4), 1101–1110. https://doi.org/10.1002/jsfa.7182.

(10) Mosbach, K. Toward the next Generation of Molecular Imprinting with Emphasis on the Formation, by Direct Molding, of Compounds with Biological Activity (Biomimetics). *Anal Chim Acta* **2001**, *435* (1), 3–8.

(11) Mosbach, K.; Ramstron, O. The Emerging Technique of Molecular Imprinting and Its Future Impact on Biotechnology. *Nat Biotechnol* **1996**, *14*, 163–170.

(12) Haupt, K.; Mosbach, K. Molecularly Imprinted Polymers and Their Use in Biomimetic Sensors. *Chem Rev* **2000**, *100* (7), 2495–2504.

(13) Piletska, E. V; Turner, N. W.; Turner, A. P. F.; Piletsky, S. A. Controlled Release of the Herbicide Simazine from Computationally Designed Molecularly Imprinted

Polymers. *Journal of Controlled Release* **2005**, *108* (1), 132–139. https://doi.org/10.1016/j.jconrel.2005.07.016.

(14) Sullivan, M. V; Dennison, S. R.; Archontis, G.; Hayes, J. M.; Reddy, S. M. Towards Rational Design of Selective Molecularly Imprinted Polymers (MIPs) for Proteins: Computational and Experimental Studies of Acrylamide Based Polymers for Myoglobin. *J Phys Chem B* **2019**, *123*, 5432–5443.

(15) Kotlarek, D.; Liu, K.; Quilis, N. G.; Bernhagen, D.; Timmerman, P.; Kouwer, P.; Dostalek, J. Thin-Film Polyisocyanide-Based Hydrogels for Affinity Biosensors. *The Journal of Physical Chemistry C* **2021**, *125* (23), 12960–12967. https://doi.org/10.1021/acs.jpcc.1c02489.

(16) Shumyantseva, V. V; Bulko, T. V; Baychorov, I. H.; Archakov, A. I. Molecularly Imprinted Polymers (MIP) in Electroanalysis of Proteins. *Biochem Mosc Suppl B Biomed Chem* **2016**, *10* (2), 145–151. https://doi.org/10.1134/S1990750816020104.

(17) Sullivan, M. V; Clay, O.; Moazami, M. P.; Watts, J. K.; Turner, N. W. Hybrid Aptamer-Molecularly Imprinted Polymer (AptaMIP) Nanoparticles from Protein Recognition-A Trypsin Model. *Macromol Biosci* **2021**, *21* (5), e2100002.

(18) Refaat, D.; Aggour, M. G.; Farghali, A. A.; Mahajan, R.; Wiklander, J. G.; Nicholls, I. A.; Piletsky, S. A. Strategies for Molecular Imprinting and the Evolution of MIP Nanoparticles as Plastic Antibodies—Synthesis and Applications. *Int J Mol Sci* **2019**, *20* (24), 6304.

(19) Shaabani, N.; Chan, N. W. C.; Lee, W. E.; Jemere, A. B. Electrochemical Determination of Naloxone Using Molecularly Imprinted Poly(Para-Phenylenediamine) Sensor. *J Electrochem Soc* **2020**, *167* (13). https://doi.org/10.1149/1945-7111/abbb0d.

(20) Sullivan, M. V; Allabush, F.; Flynn, H.; Balansethupathy, B.; Reed, J. A.; Barnes, E. T.; Robson, C.; O'Hara, P.; Milburn, L. J.; Bunka, D.; Tolley, A.; Mendes, P. M.; Tucker, J. H. R.; Turner, N. W. Highly Selective Aptamer-Molecularly Imprinted Polymer Hybrids for Recognition of SARS-CoV-2 Spike Protein Variants. *Global Challenges* **2023**, 2200215. https://doi.org/10.1002/gch2.202200215.

(21) Piletsky, S. S.; Cass, A. E. G.; Piletska, E. V; Czulak, J.; Piletsky, S. A. A Novel Assay Format as an Alternative to ELISA: MINA Test for Biotin. *ChemNanoMat* **2018**, *4* (12), 1214–1222. https://doi.org/10.1002/cnma.201800393.

(22) Singla, P.; Broughton, T.; Sullivan, M. V.; Garg, S.; Berlinguer-Palmini, R.; Gupta, P.; Smith, K. J.; Gardner, B.; Canfarotta, F.; Turner, N. W.; Velliou, E.; Amarnath, S.;

Peeters, M. Double Imprinted Nanoparticles for Sequential Membrane-to-Nuclear Drug Delivery. *Advanced Science* **2024**. https://doi.org/10.1002/advs.202309976.

(23) Kitayama, Y.; Yamada, T.; Kiguchi, K.; Yoshida, A.; Hayashi, S.; Akasaka, H.; Igarashi, K.; Nishimura, Y.; Matsumoto, Y.; Sasaki, R.; Takano, E.; Sunayama, H.; Takeuchi, T. In Vivo Stealthified Molecularly Imprinted Polymer Nanogels Incorporated with Gold Nanoparticles for Radiation Therapy. *J Mater Chem B* **2022**, *10* (35), 6784–6791. https://doi.org/10.1039/D2TB00481J.

(24) Suksuwan, A.; Lomlim, L.; Rungrotmongkol, T.; Nakpheng, T.; Dickert, F. L.; Suedee, R. The Composite Nanomaterials Containing (*R*)-thalidomide-molecularly Imprinted Polymers as a Recognition System for Enantioselective-controlled Release and Targeted Drug Delivery. *J Appl Polym Sci* **2015**, *132* (18). https://doi.org/10.1002/app.41930.

(25) Puoci, F.; Iemma, F.; Muzzalupo, R.; Spizzirri, U. G.; Trombino, S.; Cassano, R.; Picci, N. Spherical Molecularly Imprinted Polymers (SMIPs) via a Novel Precipitation Polymerization in the Controlled Delivery of Sulfasalazine. *Macromol Biosci* **2004**, *4* (1), 22–26. https://doi.org/10.1002/mabi.200300035.

(26) Xu, J.; Miao, H.; Zou, L.; Tse Sum Bui, B.; Haupt, K.; Pan, G. Evolution of Molecularly Imprinted Enzyme Inhibitors: From Simple Activity Inhibition to Pathological Cell Regulation. *Angewandte Chemie International Edition* **2021**, *60* (46), 24526–24533. https://doi.org/10.1002/anie.202106657.

(27) Piletsky, S. S.; Baidyuk, E.; Piletska, E. V.; Lezina, L.; Shevchenko, K.; Jones, D. J. L.; Cao, T. H.; Singh, R.; Spivey, A. C.; Aboagye, E. O.; Piletsky, S. A.; Barlev, N. A. Modulation of EGFR Activity by Molecularly Imprinted Polymer Nanoparticles Targeting Intracellular Epitopes. *Nano Lett* **2023**, *23* (21), 9677–9682. https://doi.org/10.1021/acs.nanolett.3c01374.

(28) Sullivan, M. V; Dennison, S. R.; Hayes, J. M.; Reddy, S. M. Evaluation of Acrylamide-Based Molecularly Imprinted Polymer Thin-Sheets for Specific Protein Capture - a Myoglobin Model. *Biomed Phys Eng Express* **2021**, 45025.

(29) Sullivan, M. V.; Nanalal, S.; Dean, B. E.; Turner, N. W. Molecularly Imprinted Polymer Hydrogel Sheets with Metalloporphyrin-Incorporated Molecular Recognition Sites for Protein Capture. *Talanta* **2024**, *266*, 125083. https://doi.org/10.1016/j.talanta.2023.125083.

(30) Blackburn, C.; Sullivan, M. V.; Wild, M. I.; O' Connor, A. J.; Turner, N. W. Utilisation of Molecularly Imprinting Technology for the Detection of Glucocorticoids for a Point of Care Surface Plasmon Resonance (SPR) Device. *Anal Chim Acta* **2024**, *1285*, 342004. https://doi.org/10.1016/j.aca.2023.342004.

(31) Hawkins, D. M.; Stevenson, D.; Reddy, S. M. Investigation of Protein Imprinting in Hydrogel-Based Molecularly Imprinted Polymers (Hydrogels). *Anal Chim Acta* **2005**, *542* (1), 61–65.

(32) Chalmers, J. M.; Everall, N. J.; Ellison, S. Specular Reflectance: A Convenient Tool for Polymer Characterization by FTIR-Microscopy. *Micron* **1996**, *27*, 315–328.

(33) Sammon, C.; Yarwood, J.; Everall, N. An FT-IR Study of the Hydrolytic Degradation on the Structure of Thin PET Films. . *Polym Degrad Stab* **2000**, *67*, 149–158.

(34) Sammon, C.; Mura, S.; Yarwood, J.; Everall, N.; Swart, R.; Hodge, D. FTIR-ATR Studies of the Structure and Dynamics of Water Molecules in Polymeric Matrixes. A Comparison of PET and PVC. *Journal of Physical Chemistry B* **1998**, *102*, 3402–3441.

(35) Zayats, M.; Brenner, A. J.; Searson, P. C. Protein Imprinting in Polyacrylamide-Based Gels. *Biomaterials* **2014**, *35* (30), 8659–8668. https://doi.org/10.1016/j.biomaterials.2014.05.079.

(36) Ur Rehman, H.; Nawaz, M. A.; Pervez, S.; Jamal, M.; Attaullah, M.; Aman, A.; Ul Qader, S. A. Encapsulation of Pectinase within Polyacrylamide Gel: Characterization of Its Catalytic Properties for Continuous Industrial Uses. *Heliyon* **2020**, *6* (8), e04578. https://doi.org/10.1016/j.heliyon.2020.e04578.

(37) Kimhi, O.; Bianco-Peled, H. Study of the Interactions between Protein-Imprinted Hydrogels and Their Templates. *Langmuir* **2007**, *23* (11), 6329–6335.

(38) Sissons, J. W. Digestive Enzymes of Cattle. *J Sci Food Agric* **1981**, *32* (2), 105–114. https://doi.org/10.1002/jsfa.2740320202.

(39) Ketrat, S.; Japrung, D.; Pongprayoon, P. Exploring How Structural and Dynamic Properties of Bovine and Canine Serum Albumins Differ from Human Serum Albumin. *J Mol Graph Model* **2020**, *98*, 107601. https://doi.org/10.1016/j.jmgm.2020.107601.

(40) Kuddus, M. Introduction to Food Enzymes. In *Enzymes in Food Biotechnology*; Elsevier, 2019; pp 1–18. https://doi.org/10.1016/B978-0-12-813280-7.00001-3.

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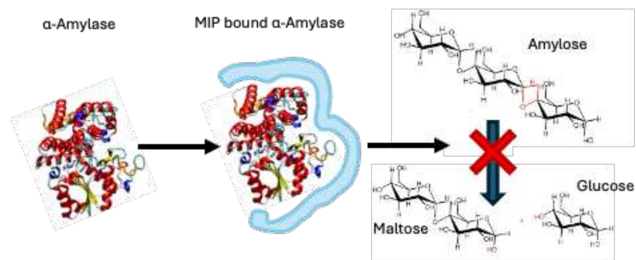

Inhibition of α-Amylase using molecularly imprinted polymers (MIPs) preventing the digestion of starch into sugars