Hierarchically imprinted polymer for peptide tag recognition based on an oriented surface epitope approach

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ABSTRACT: FLAG tag (DYKDDDDK) is a short peptide commonly used for the purification of recombinant proteins. The high price of the affinity columns and their limited reusability are a shortcoming for their widespread use in biotechnology applications. Molecularly imprinted polymers (MIPs) can circumvent some of the limitations of bioaffinity columns for such application, including long-term stability, reusability, and cost. We report herein the synthesis of MIPs selective to the FLAG tag by hierarchical imprinting. Using the epitope imprinting approach, a 5-aminoacid peptide DYKDC was selected as template and was covalently immobilized on the surface of microporous silica beads, previously functionalized with different aminosilanes namely, 3-(2-aminoethylamino)propyldimethoxymethylsilane, AEAPMS, and *N*-(2-aminoethyl)-2,2,4-trimethyl-1-aza-2-silacyclopentane, AETAZS. We investigated the effect of the type of silane on the production of homogeneous silane grafted layers with the highest extent of silanol condensation as possible using ²⁹Si CP/MAS NMR. We observed that the right orientation of the imprinted cavities can substantially improve analyte recoveries from the MIP. After template and silica removal, the DYKDC-MIPs were used as sorbents for solid-phase extraction (molecularly imprinted solid-phase extraction) of the FLAG peptide, showing that the polymer prepared with AETAZS-bound silica beads contained binding sites more selective to the tag ($R_{\text{MIP-AZA}} = 87.4\%$ vs $R_{\text{NIP-AZA}} = 4.1\%$, $n = 3$, $\text{RSD} \leq 4.2\%$) than those prepared using AEAPMS ($R_{\text{MIP-DM}} = 73.4\%$ vs $R_{\text{NIP-DM}} = 23.2\%$, $n = 3$, $\text{RSD} \leq 4.0\%$) as functionalization agent. An extensive computational molecular modeling study was also conducted, shedding some light on the interaction mechanism between the FLAG peptide and the imprinted template in the binding cavities.

Keywords: epitope imprinting, FLAG tag, hierarchically imprinted polymer, silane grafting, computational modeling

INTRODUCTION

The advent of fusion tags marked a milestone in biotechnology allowing protein purification on demand. These affinity tags are short polypeptides expressed together with the target protein by recombinant DNA technology. Its further purification is based mainly on the interaction between specific antibodies and the fused peptidic sequence. Despite the commercial availability of immunoaffinity resins and their variety, the production of antibody receptors is time-consuming and expensive. Therefore, a broad palette of peptide tags has emerged, resulting in a wide diversity of different sequences, sizes, and features of application in different fields.¹

FLAG tag is a versatile purification peptide composed of eight aminoacids (DYKDDDDK) specifically designed by Hopp et al.² for immunopurification chromatography. Because of its hydrophilic nature, the tag is located on the surface of the

recombinant protein, increasing the solubility of the fusion partner to achieve higher purity values. Moreover, this polypeptide does not interfere with the structure and the function of the fused protein and can be used in different expression systems.³ It can be applied as a single peptide or in a combination of three consecutive repetitions (3xFLAG).⁴ FLAG-tagged proteins are purified using anti-FLAG monoclonal antibodies (M1 or M2) immobilized on agarose resins or on magnetic nanoparticles.⁵ Although this immunopurification procedure is highly selective, the limited binding capacity, reusability and high cost of these materials have prompted the search of new purification alternatives.⁶

Molecularly imprinted polymers (MIPs) are biomimetic synthetic receptors intrinsically more stable and robust than antibodies with significantly higher reusability (see Table S1, Supporting Information).⁷ These polymeric

Figure 1. (a) Derivatization of silica particles: (1) silanization of rehydroxylated silica beads by using two types of silane agents: AEAPMS (3-(2-aminoethylamino) propyldimethoxyme-thylsilane) and AETAZS (*N*-(2-aminoethyl)-2,2,4-trimethyl-1-aza-2-silacyclopentane), (2) iodoacetylation of amino-modified silica particles, (3) peptide DYKDC coupling through the thiol group of the C-terminus cysteine. (b) Hierarchically imprinted polymer synthesis: (1) polymerization using EAMA (*N*-(2-aminoethyl)methacrylamide hydrochloride) as a functional monomer and ethyleneglycol dimethacrylate (EDMA) as a cross-linker, (2) template and silica removal, and (3) FLAG recognition by epitope-imprinted cavities. (c) Chemical structures of FLAG (DYKDDDDK), DYKDC and DYKD peptides, where D= aspartic acid, Y= tyrosine, K= lysine and C= cysteine.

materials display selective cavities generated during polymerization because of the formation of pre-polymerization complexes between the template molecule and the selected functional monomers. A three dimensional polymer network is built around this complex by the addition of a cross-linking agent. Subsequent template removal leaves specific binding sites complementary in terms of size, shape, and geometry of functional groups with the template molecule, displaying a “memory effect”.^{8,9} Their synthesis has been extended to almost any kind of template, but macromolecular templating still remains a challenge because of the big size, complexity, conformational flexibility, and limited solubility of these biomolecules.¹⁰ Epitope imprinting appears to be a suitable alternative to circumvent these drawbacks.^{11,12} This approach is based on the use of a short peptide (known as epitope) that represents a larger peptide or protein, so the final MIP will be able to recognize both structures.^{13,14} Nevertheless, to ensure high selectivity of the imprinted material to the target molecule, the

epitope must be carefully selected as the polymer selectivity will depend of the uniqueness of its sequence.^{15,16}

Despite the epitope imprinting popularity,^{17,18,19} the use of commercial affinity tags as template molecules for the preparation of imprinted materials able to compete with commercial resins is still challenging. Ideal imprinted materials should display controlled particle size, shape, and porosity, and consequently, spherical particles are preferred to bulk materials. Imprinted polymers prepared by the hierarchically imprinting strategy combine all of these features by using porous silica beads as sacrificial supports.²⁰ The template molecule is immobilized in the pores of the particles and subsequently filled with the prepolymerization mixture. After polymerization, the template-grafted silica beads are dissolved, leaving the “negative image” of the templated scaffold.²¹ The imprinted material will contain binding sites oriented and located at the surface, exhibiting more accessible open-entrance cavities for analyte recognition and faster adsorption kinetics.^{22,23}

In a previous work, we developed a nonhierarchically imprinted polymer also based on the epitope approach by using the peptide DYKD as template molecule.²⁴ Despite the good results obtained with the former MIP, the recognition of the FLAG peptide showed significant nonspecific binding when compared with the nonimprinted polymer (NIP). Thus, in order to suppress this effect, we aim to prove in this work the known effectiveness of the hierarchically imprinting strategy by using the template orientation.

Herein we report the synthesis of a hierarchically imprinted polymer combined with the aforementioned epitope approach for the recognition of the FLAG tag using the peptide DYKDC as template molecule. This pentapeptide was immobilized into the pores of iodoacetylated silica microbeads through the thiol group of the C-terminus cysteine, preceded by an initial amino modification step. The functionalization process involves a thorough control of the synthetic conditions to avoid the formation of multiple silane layers that would lead to a possible clogging of the silica pores and thus an incomplete filling of these pores with the prepolymerization mixture. In this regard, two types of aminosilane agents were evaluated to achieve a homogeneous monolayer surface coverage of the inorganic support. Derivatized silane-grafted particles were thoroughly investigated by solid-state NMR to elucidate the silane moiety distribution on the silica beads. Based on our previous work²⁴, hierarchically imprinted polymers were also prepared using EAMA as the functional monomer in combination with EDMA as cross-linking agent (Table S2, Supporting Information). Interestingly, the hierarchically imprinted beads prepared with AETAZS as a coupling agent in the initial derivatization step show an enhanced binding behavior toward the FLAG peptide. To further understand this outstanding and specific recognition performance of the FLAG peptide toward the imprinted cavities, we performed an extensive computational molecular modeling study of the free peptides (DYKD and FLAG) and the complex between the immobilized template DYKDC and the functional monomer EAMA.

RESULTS AND DISCUSSION

Derivatization of silica beads. Functionalization of silica particles with the peptide DYKDC has been accomplished in several steps. A general overview of the strategy is depicted in Figure 1. Amino groups were introduced by using two different silanization agents: AEAPMS or AETAZS, see Figure S2, Supporting Information. These aminated beads were then modified by introducing iodoacetamide groups, able to react in the subsequent step with the thiol group of the C-terminus cysteine in the template peptide DYKDC. The amount of immobilized ligand (expressed as degree of functionalization in mmol g⁻¹, D_f) on the silica surface was estimated by elemental microanalysis data, more precisely from the change in carbon content ($\Delta\%C$) compared by measuring the preceding step (eq S1, Supporting Information).²⁵ As silanization of silica beads is the most crucial step in the functionalization procedure, amino-functionalized particles were subjected to additional analysis to elucidate the coupled ligand distribution. An uncontrol grafting reaction and/or an inappropriate choice of the silane will lead to a complete or partial pore blockage by multilayer formation, avoiding the proper polymer synthesis. Thus, different reaction conditions were investigated in order to achieve a homogeneous surface silane coverage.

Silica bead functionalization using AEAPMS. The ideal scenario for further functionalization of the silica particles would be a monolayer grafting of the silane along the silica surface with the highest extent of silanol condensation as possible. With this purpose, different AEAPMS concentration were tested (3.75 mmol, 7.5 mmol or 11.25 mmol g⁻¹). As described previously by García et al.²⁶ the use of *p*-toluenesulfonic acid (PTSA) accelerates the silane hydrolysis reaction close to the silica surface and then the condensation with surface silanols, acting this compound as a catalyst. As shown in Table S3 (Supporting Information), as silane AEAPMS concentration increases, the functionalization degree (D_f) increases slightly, rendering 0.24 (Si-DMPTSA1), 0.27 (Si-DMPTSA2) and 0.28 mmol g⁻¹ (Si-DMPTSA3) immobilized on the surface, respectively. However, an increase in silane concentration did not produce a significant improvement in their D_f .

The ²⁹Si CP/MAS NMR spectra of samples Si-DMPTSA1, Si-DMPTSA2, and Si-DMPTSA3 are included in Figure 2. The spectra show the presence of two groups of signals assigned to the inorganic Q region at the highest field and the organic D region assigned to the silane-grafted moieties.²⁷ These regions are, in their turn, decomposed into different signals accounting for the condensation degree of the silyl species involved. The main difference between the spectra is the lower intensity of the Q3 signal ($\sim$ 102 ppm), assigned to free silanols onto the silica surface, in sample Si-DMPTSA1 compared to the other two samples. This suggests a higher surface coverage by silane grafting in this former sample. This means that, although the silane content in the derivatization products increases with the silane concentration, the extent of silanol condensation on the surface does not change or it is even lower when the concentration of the silanization agent increases. This is probably due to an increase in the self-condensation of amino silanes in the concentrated media before grafting on the surface. Within the scope of this work, the grafting of condensed silane species (brushes as the silane agent is difunctional) is not desirable because they can block the silica pores. Thus, 3.75 mmol g⁻¹ of AEAPMS was selected as optimum concentration for further steps.

For a complete optimization method, the reaction time and temperature were also investigated under the optimum AEAPMS concentration. Longer reaction times (up to 16h as in Si-DMPTSA4 sample) yield a higher functionalization degree (Table S3, Supporting Information). However, the absence of catalyst (Si-DMRT) results in a decrease of the grafting content. Figure S3 (Supporting Information) shows the ²⁹Si CP/MAS NMR spectra of the samples Si-DMPTSA4 and Si-DMRT. Si-DMPTSA1 spectrum was included for the sake of comparison. This latter sample is, again, the one with the lowest intensity of the Q3 signal, which indicates a higher extent in the silanol condensation under these reaction conditions. On the other hand, the sample Si-DMPTSA4 shows the highest intensity of the D signal according to its higher content of grafted silane. It seems that as the reaction is prolonged in time, the self-silane condensation is favored instead of increasing the surface silanol reaction. Actually, Si-DMPTSA4 sample exhibits a D signal sharper and located closer to -22 ppm, where siloxane units in the liquid state (i.e. far from the silica surface) appear.²⁷ Therefore, the Si-DMPTSA4 spectrum is consistent with long silane brushes grafted to the silica surface, whereas Si-DMPTSA1 would have a grafting coverage

close to a monolayer structure onto the silica surface. From these results, the reaction conditions of sample Si-DMPTSA1 were chosen as the most suitable for within the scope of this work.

Figure 2. ^{29}Si CP/MAS NMR spectra of silica particles functionalized with silane AEAPMS using different conditions: Si-DMPTSA1 (3.75 mmol g^{-1} , 110 $^{\circ}\text{C}$, PTSA, 2 h), Si-DMPTSA2 (7.5 mmol g^{-1} , 110 $^{\circ}\text{C}$, PTSA, 2 h), and Si-DMPTSA3 (11.25 mmol g^{-1} , 110 $^{\circ}\text{C}$, PTSA, 2 h).

These results are consistent with a study published by Smith and Chen,²⁸ which proves by using different silanization experiments that silane layers prepared in anhydrous medium at elevated temperatures possessed higher functional group density and exhibited greater hydrolytic stability than those prepared in vapor phase or at room temperature. Reproducibility of the procedure at optimum reaction conditions (Si-DMPTSA1) was evaluated by synthesizing three different batches obtaining a mean D_f of 0.26 mmol g^{-1} (RSD $\leq 12\%$) proving a good replicability of the synthesis procedure (see Table S4, Supporting Information). These data rely also on porosity studies evaluated from N_2 adsorption/desorption analysis. Figure S4 (Supporting Information) shows a comparison of the isotherms measured using commercial silica particles and sample Si-DMPTSA1 showing a type IV curve, indicating that the pore size of the derivatized silica is located in the mesopore range, according to the IUPAC classification.²⁹ Results revealed a decrease in the specific surface area (S_A) and the specific pore volume (V_p) for the amino-modified beads (Si-DMPTSA1) obtaining a S_A of 40 $\text{m}^2 \text{g}^{-1}$ and V_p of 0.64 $\text{cm}^3 \text{g}^{-1}$ compared with the commercial silica particles (S_A of 56 $\text{m}^2 \text{g}^{-1}$ and V_p of 0.71 $\text{cm}^3 \text{g}^{-1}$). These results support the presence of the silane moieties attached to the surface of the bead, as well as the suitability of the synthesis procedure as the pore volume is reduced less than 10%.

Silica bead functionalization using AETAZS. For comparison, AETAZS was also used as silanization agent. This silane cannot self-condense, so the reaction produces a monolayer onto the silica surface. The optimization of the reaction conditions pursued the highest extent of silanol condensation. Optimization of AETAZS cyclic azasilane route was evaluated at three concentration levels (3.75, 7.5 mmol or 11.25 mmol g^{-1}), showing a mild increase in carbon content as AETAZS concentration increases (see Supporting Information, Table S5).

Nevertheless, there are not significant differences in the D_f between samples, as described previously for AEAPMS, rendering 0.12 (Si-AZA1), 0.13 (Si-AZA2), and 0.13 mmol g^{-1} (Si-AZA3), respectively.

Surface analysis by ^{29}Si CP/MAS NMR reveals almost an identical structure pattern for the three samples (see Figure S5, Supporting Information). Because there are no significant differences in silane content and surface silanol condensation extent, the lowest silane concentration in the reaction medium was preferred. Three different batches were synthesized at optimum silane concentration (Si-AZA1) obtaining a mean D_f of 0.14 mmol g^{-1} (RSD $\leq 8\%$), showing an excellent synthesis reproducibility (see Table S6, Supporting Information). Nitrogen sorption analysis (Figure S6, type IV curve) revealed the same specific surface area as for Si-DMPTSA1 (40 $\text{m}^2 \text{g}^{-1}$) but a higher specific pore volume (0.69 $\text{cm}^3 \text{g}^{-1}$) that would explain the lower functionalization degree and a better adsorbability.³⁰ The pore size distribution curves obtained from N_2 adsorption and desorption isotherms for the Si-AZA1, Si-DMPTSA1, and rehydroxylated silica (SiOH) samples are shown in Figures S7a,b (see Supporting Information).

Although at a glance there are no substantial changes in the surface of both amino-functionalized particles under the microscope (see Figure S8, Supporting Information), the main differences can be observed between them after the coupling of the silane agent used in the derivatization process. The AEAPMS silane includes two methoxy groups that could condense with one or two groups in the silanol moieties, while the AETAZS silane can only be attached by one arm by performing a ring-opening reaction (see Figure S2, Supporting Information). This fact leads to a more targeted reaction, where AETAZS acts as a monovalent reagent avoiding self-condensation processes. Moreover, the synthesis of three different batches revealed the use of the cyclic azasilane as a more reproducible method (RSD $\leq 8\%$) compared with the dimethoxy silanization (RSD $\leq 12\%$). The use of the azacompound is not very popular^{31,32,33} because most of the published studies report the application of di- or trimethoxy/ethoxy silanes (i.e. APTES, 3-aminopropyltrimethoxysilane).³⁴ However, our results prove that this coupling agent is an excellent selection when the formation of a monolayer and high grafting yields are sought.

In peptide DYKDC coupling, amino-modified silica particles were iodoacetylated in order to introduce an iodine atom for further substitution by the thiol group of the peptide (Figure S9, Supporting Information). In the case of the iodoacetylated Si-DMPTSA1 particles (Si-DM-IAC), the obtained D_f was 0.15 mmol g^{-1} (RSD $\leq 18\%$) whereas in the case of the Si-AZA1 derivatized particles (Si-AZA-IAC) D_f was 0.10 mmol g^{-1} (RSD $\leq 16\%$) (see Tables S7 and S8, Supporting Information). By applying eq S2 (Supporting Information), the coupling yield can be evaluated toward the preceding step, rendering 58% for Si-DM-IAC and 71% for Si-AZA-IAC. This higher yield obtained for the aza-silane beads can be associated with the lower steric hindrance of the azasilane moieties grafted onto the silica surface.

Template immobilization is a critical step in the hierarchically imprinting strategy for the proper performance of the final imprinted polymer. A high constrained surface coverage may lead to a template aggregation and consequently, an inef-

ficient formation of binding sites. On the contrary, a low template coverage will render insufficient binding sites and therefore the material will lose the ability of the selective recognition. Peptide DYKDC was coupled to the corresponding iodoacetylated silica beads (Si-DM-IAC or Si-AZA-IAC) through the C-terminus cysteine thiol taking advantage of the selectivity of this mercapto group (see Figure S10, Supporting Information).³⁵ Similar results were obtained for both functionalization reactions in terms of reaction yield ($\sim 35\%$) and a close mean functionalization degree of 0.05 mmol g^{-1} ($\text{RSD} \leq 8\%$) for Si-DM-Pept and $0.035 \text{ mmol g}^{-1}$ ($\text{RSD} \leq 9\%$) for Si-AZA-Pept (Tables S9 and S10, Supporting Information). These functionalization results are in agreement with those obtained by other authors using the silica templating strategy. For example, Wierzbicka et al.³⁶ reported the immobilization of phosphotyrosine using amino-functionalized silica beads ($30 \mu\text{m}$ particle size, $45 \text{ m}^2 \text{ g}^{-1}$ surface area and 47.5 nm pore diameter) as well. They obtained a maximum template functionalization of $0.055 \text{ mmol g}^{-1}$ comparable to the peptide immobilization reported in this work.

Synthesis of the hierarchically imprinted polymer. The epitope imprinting approach has been recognized as an effective strategy for the synthesis of imprinted polymers selective to proteins.³⁷ In a previous study, we demonstrated the use of the N-terminus four-amino acid epitope of the FLAG tag (DYKD) as a template molecule for the preparation of MIPs useful for the purification of FLAG-tagged proteins.²⁴ The resulting MIP (denoted as MSP8, 0.5:3:20 mole ratio DYKD/EAMA/EDMA) aimed to mimic the binding pocket of the ANTI-FLAG M2 monoclonal antibody^{38,39} and showed a good performance in the extraction of both the FLAG- and DYKD-tagged proteins. However, the nonspecific binding of the FLAG tag in the MSP8 polymer was relatively high with an IF in the order of 3.3.

In order to improve the MIP performance and reduce the nonspecific binding for FLAG tag purification, we decided to explore the hierarchically imprinting approach. The peptide DYKDC was covalently immobilized on the iodoacetylated silica beads, as described previously, and EAMA and EDMA were used as functional and cross-linking monomers, respectively, for polymer synthesis. Dimethyl sulfoxide (DMSO) was used as porogen. In contrast to traditional imprinting strategies (i.e., bulk imprinting), the preparation of spherical polymer particles allows the preparation of MIPs with a better mass transfer properties and higher superficial areas. The use of the functionalized silica microbeads ($40\text{--}75 \mu\text{m}$ diameter, $500 \text{ \AA}$ pore size) as sacrificial scaffolds in combination with the hierarchically imprinting strategy should lead, in principle, to the preparation of MIPs with improved recognition properties. After silica etching and template removal, the binding sites of the resulting MIP particles should be on the surface of the material favoring the diffusion of the analytes into the binding sites in the rebinding step.²⁰ These materials can be considered as “mirror image” structures of the silica used as a support (Figure 1b).⁴⁰

To maintain the desired spherical structure in the MIP, the prepolymerization mixture should fully penetrate into the pores of the silica. Nonetheless, the use of an excess of the prepolymerization solution will lead to a complete pore blockage in the resulting composite, causing low efficiencies for the targeted application.⁴¹ The volume of the prepolymerization

mixture used for filling each silica sample was calculated considering 95% of the pore volume obtained in the nitrogen sorption analysis for the corresponding silanized silica.⁴²

Peptide-functionalized silica beads play a double role in polymer synthesis since they serve simultaneously as template and sacrificial scaffold in the hierarchically imprinting approach. Imprinted polymers were synthesized using the functionalized supports Si-DM-Pept and Si-AZA-Pept, designating the imprinted organic networks as MIP-DM and MIP-AZA, respectively. The molar ratios between immobilized template (T), functional monomer (FM) and cross-linking agent (CL) were of 0.6:4:20 for MIP-DM and 0.4:4:24 in the case of the MIP-AZA (detailed composition in Table S2, Supporting Information). In order to evaluate nonspecific interactions, a non-imprinted material (NIP) was synthesized with all the cocktail components but in the absence of the template molecule. The iodoacetylated silica beads (Si-DM-IAC and Si-AZA-IAC) were selected as scaffolds for NIP preparation, designed as NIP-DM and NIP-AZA, respectively. Figure 3 shows the SEM micrographs of the peptide-functionalized silica beads and the corresponding hierarchically imprinted polymers. The sphericity of the particles is maintained after the polymerization step (Figure S11, Supporting Information), which is critical to achieve an efficient packing in the solid-phase extraction (SPE) cartridges. The MIP particles exhibit a more irregular surface than the silica molds with a porous structure that facilitates the access of the analyte to the binding sites in the polymer.

Figure 3. Scanning electron micrographs of the peptide-derivatized silica beads Si-DM-Pept (a) and Si-AZA-Pept (b) and the corresponding hierarchically imprinted polymers MIP-DM (c) and MIP-AZA (d) and NIP-DM (e) and NIP-AZA (f).

Evaluation of the retention of the DYKD and FLAG peptides by Molecularly Imprinted SPE. The MIP-DM and MIP-AZA particles (25 mg), and their corresponding NIPs, were packed in 1 mL SPE cartridges. After conditioning with HEPES buffer (100 mM , $\text{pH } 7.5$), 1 mL of aqueous solutions of DYKD and FLAG peptides at 5 mg L^{-1} were percolated through the cartridges. A washing step using water (0 , 1 , or 3 mL) was introduced in order to remove the nonspecific interactions. Finally, the peptides were eluted with 1 mL of tetrabutylammonium hydrogensulfate (TBA) 1% (w/v) to disrupt the interactions between the polymer and peptides.

Recoveries obtained for the DYKD epitope decreased in both imprinted and NIPs with increasing water washing volumes, revealing a nonspecific binding behavior of the imprinted cavities (Figure 4a). Recoveries from the hierarchically imprinted polymers, without washing step ($R_{\text{MIP-DM/DYKD}} = 52.6\%$, $\text{RSD} \leq 6.1\%$; $R_{\text{MIP-AZA/DYKD}} = 64.7\%$, $\text{RSD} \leq 5.4\%$), are comparable to those obtained using nonhierarchically DYKD-imprinted MIPs (designed as MSP8) and 3 mL of water as washing solvent ($R_{\text{MSP8/DYKD}} = 64\%$; $\text{RSD} \leq 7\%$). However, the recoveries from the hierarchically NIPs ($R_{\text{NIP-DM/DYKD}} = 22.9\%$, $\text{RSD} \leq 6.3\%$; $R_{\text{NIP-AZA/DYKD}} = 37.1\%$, $\text{RSD} \leq 5.3\%$) are considerably higher than for the NSP8 ($R_{\text{NSP8/DYKD}} = 5\%$; $\text{RSD} \leq 1\%$). It can be noticed that the retention of DYKD in the MIPs dramatically decreases as the washing volume increases, indicating a low retention in the polymer.

Figure 4. MISPE recoveries (%; $n=3$, RSD : 2-8%) from imprinted (MIP-DM and MIP-AZA) and NIPs (NIP-DM and NIP-AZA) after percolating 1 mL of (a) DYKD and (b) FLAG at 5 mg L^{-1} each, HEPES buffer (100 mM, pH 7.5) applying different washing volumes of water (0, 1, and 3), and elution with 1 mL water/TBA 1% (w/v).

Regarding the retention of FLAG, the differences between the MIP and the NIP are larger in all cases than those observed with DYKD (Figure 4b). As the washing volume increases (0–3 mL water), the recoveries for the FLAG in the MIP-DM decrease from 97.2% ($R_{\text{MIP-DM/FLAG/0-mL}}$; $\text{RSD} \leq 6.8\%$) to 73.4% ($R_{\text{MIP-DM/FLAG/3-mL}}$; $\text{RSD} \leq 3.8\%$), whereas in the NIP-DM, the recoveries decreased from 36.5% ($R_{\text{NIP-DM/FLAG/0-mL}}$; $\text{RSD} \leq 5.4\%$) to 23.2% ($R_{\text{NIP-DM/FLAG/3-mL}}$; $\text{RSD} \leq 4\%$). These recovery values were similar to those obtained using the MSP8 polymer and 3 mL of washing solvent volume ($R_{\text{MSP8/FLAG}} = 89\%$; $\text{RSD} \leq 6\%$; $R_{\text{NSP8/FLAG}} = 27\%$; $\text{RSD} \leq 4\%$). Recoveries of FLAG are higher from the MIP-AZA particles, and the differences between the MIP and the NIP were also higher. Without the washing step, the FLAG recovery is almost quantitative in the MIP ($R_{\text{MIP-AZA/FLAG/0-mL}} = 99.8\%$; $\text{RSD} \leq 3.2\%$) and relatively low in the NIP ($R_{\text{NIP-AZA/FLAG/0-mL}} = 19.6\%$; $\text{RSD} \leq 5.4\%$). By using 3 mL of washing volume, differences between MIP and NIP are even more noticeable ($R_{\text{MIP-AZA/FLAG/3-mL}} = 87.4\%$; $\text{RSD} \leq 4\%$ vs. $R_{\text{NIP-AZA/FLAG/3-mL}} =$

4.1% ; $\text{RSD} \leq 4.2\%$), obtaining an imprinting factor (IF) value of 21.3 (calculated as $R_{\text{MIP}}/R_{\text{NIP}}$, see Table S11). This data is considerably higher than the value reported previously (IF of 3.3)²⁴, highlighting the success of the epitope imprinting effect.

The differences between MIP-DM and MIP-AZA resemble a typical monoclonal antibody performance in immunoassays, where an excess of antibody leads to a higher nonspecific binding. As the amount of peptide immobilized in the Si-AZA-Pept ($0.035 \text{ mmol g}^{-1}$) is lower than for the Si-DM-Pept (0.05 mmol g^{-1}), it is expected that the peptide binding sites in the MIP-AZA polymer are less and more homogeneously distributed than in the MIP-DM resulting in better defined cavities.

In view of these results, the MIP-AZA was used in MISPE and loaded with different concentrations (1, 10 and 25 mg L^{-1}) of DYKD and FLAG peptides in order to evaluate their retention performance (Figure 6). In the case of the DYKD peptide, retention in both the MIP and the NIP decreases significantly with the loading concentration and the washing volume.

Figure 5. MISPE recoveries (%; $n = 3$, RSD : 1-7%) from MIP-AZA (●) and NIP-AZA polymers (▲) after percolating 1 mL of peptide solutions of (a) DYKD and (b) FLAG at different concentration levels (1, 5, 10 and 25 mg L^{-1}) applying various washing volumes of water (0, 1, and 3 mL) and elution with 1 mL water/TBA 1% (w/v).

However, the retention of the FLAG in the MIP-AZA is always significantly higher in MIP compared to NIP (Figure 5b), and slightly decreases as both concentrations of peptide and washing volumes increase. It is worth mentioning that the IFs in the tested conditions are always significantly higher to those reported previously.²⁴ These results strongly confirm the recognition ability of the MIP-AZA for the effective retention of FLAG peptide, reinforcing the usefulness of the epitope imprinting approach as a synthetic strategy for MIP synthesis.⁴³ These results are in agreement with those reported by Rachkov and Minoura,¹³ showing that a polymer synthesized using a tetrapeptide as a template molecule (YPLG) exhibited

a stronger retention for the longer nonapeptide oxytocine (CYIQNCPLG) under certain conditions.

Cross-reactivity assays. The affinity of the MIP-AZA to other peptides GRGDNP and YPYDVDPYA was evaluated using the optimized MISPE procedure (see Supporting Information, Section S5). Peptide GRGDNP includes an aspartic acid in same position as DYKD and FLAG (DYKDDDDK). Recoveries obtained for this peptide were negligible and similar for both MIP and NIP ($R_{\text{MIP-AZA/GRGDNP}} = 7.6\%$, $\text{RSD} \leq 2.1\%$; $R_{\text{NIP-AZA/GRGDNP}} = 7.5\%$, $\text{RSD} \leq 7.4\%$). The second peptide (YPYDVDPYA, known as HA peptide) was chosen by virtue of its usefulness as fusion tag in protein purification processes. This nine-amino acid polypeptide derives from the human influenza hemagglutinin (HA) protein and is commonly used as protein purification partner by using HA-tag specific antibodies. The peptide contains several aspartic acids and tyrosine amino acids in its structure. Recoveries obtained were also negligible ($R_{\text{MIP-AZA/HA}} = 8.6\%$, $\text{RSD} \leq 10.5\%$; $R_{\text{NIP-AZA/HA}} = 2.3\%$, $\text{RSD} \leq 10.2\%$). The selectivity achieved in this approach can be attributed to the high quality of the binding sites generated on the surface of the hierarchically imprinted polymer. Whereas in other methods (i.e., bulk imprinting), the binding cavities can be occluded, generating a random distribution of binding sites with different affinities as it happens with polyclonal antibodies.⁴⁴ Template-forced orientation leads to more accessible open-access cages for analyte recognition.⁴⁵ This fact has a direct effect on the recognition properties of the material as we have demonstrated for the FLAG peptide using the MIP-AZA polymer. This behavior is well known for monoclonal antibodies, where affinity increases with a decrease in the conformational flexibility of the antigen binding site of the antibody.⁴⁶

Molecular Dynamics Simulations. To gain better understanding of why the hierarchically MIP templated with the peptide DYKDC recognizes better the FLAG peptide (DYKDDDDK) than the shorter DYKD peptide, we performed extensive molecular dynamics (MD) simulations of: 1) DYKD peptide (1 μs), 2) DYKDDDDK peptide (1 μs); and 3) DYKDC peptide in complex with EAMA (1.2 μs and the analysis was conducted on the last 1 μs MD simulation). We analyzed each of the three simulations using root-mean-square deviation (rmsd) and two-dimension-RMSD (2D-rmsd).

The MD simulation of DYKDC peptide in complex with EAMA exhibits mainly two different conformations for the peptide: (1) the semiextended (linear) one forms an S-like shape with a population of $\sim 60\%$ (average $\sim 0.8 \pm 0.3$ Å all peptide residues rmsd with respect to equilibrated structure backbone atoms; see Figure 6); and (2) the other one forms a turn starting at Y2 toward the C-terminus with a population of $\sim 40\%$ (average $\sim 2.0 \pm 0.2$ Å rmsd; see Figure 6 depicting the last 1 μs MD simulation). The analysis of the 2D-rmsd shown in Figures 6b and S12a (Supporting Information) confirms the formation of the two above-mentioned conformations. There is no difference between the rmsd and 2D-rmsd graphs since the conformational changes occurred in block during the MD simulation (see Figures 6b,c) contrary to the DYKDDDDK peptide behavior as described below. EAMA stabilized after 22 ns MD simulation displaying two main populations (average $\sim 3.7 \pm 1.9$ Å and average $\sim 6.8 \pm 1.2$ Å) with an overall all ligand heavy atoms' average rmsd of $\sim 5.5 \pm 2.2$ Å with respect

to the equilibrated structure backbone atoms (see Figure 6a and S13, Supporting Information).

As shown in Figure 6c, the main interactions for cluster 1's and cluster 2's representative structures, respectively, are mainly through three hydrogen bonds between the hydrogen of $-\text{NH}_3^+$ (i.e., two salt bridges) and $-\text{NH}$ groups of EAMA and oxygen of $-\text{COO}^-$ group of D4 and C5 highlighted with dashed lines (see Tables S12-S14, Supporting Information).

Figure 6. Computational study of DYKDC peptide/EAMA complex in DMSO. (a) rmsd with respect to equilibrated structure backbone atoms; last 1 μs MD simulation. (b) Left panel: two-dimensional rmsd; right panel: cluster analysis: the two main representative conformations are depicted – Cluster 1 (blue, $\sim 40\%$), and Cluster 2 (orange, $\sim 60\%$). (c) Main interactions for cluster 1's (left panel) and cluster 2's (right panel) representative structures, respectively. Hydrogen bonds between the $-\text{NH}_3^+$ (i.e. two salt bridges) and $-\text{NH}$ groups of EAMA and $-\text{COO}^-$ groups of D4 and C5 respectively are shown using dashed lines.

On the other hand, as shown in Figure 7 and S12b (Supporting Information), the DYKD peptide MD simulation predicts three different conformations: (1) the semiextended (linear) one forms an S-like shape with a population of $\sim 50\%$ (average $\sim 0.7 \pm 0.1$ Å all peptide residues rmsd with respect to the minimized initial structure backbone atoms); (2) the other one forms a turn starting at Y2 toward the C-terminus with a population of $\sim 37\%$ (average $\sim 1.1 \pm 0.2$ Å rmsd); and (3) the remaining one forms a horseshoe-like shape with a population of

~13% ($\sim 2.2 \pm 0.3$ Å rmsd). The all-residues peptide average rmsd including the three clusters is $\sim 1.1 \pm 0.5$ Å along the MD simulation.

Figure 7. Computational study of DYKD peptide in water. (a) rmsd of DYKD peptide with respect to minimized initial structure backbone atoms. (b) Left panel: two-dimensional rmsd; right panel: cluster analysis: three representative conformations are depicted – cluster 1 (orange, ~50%), cluster 2 (blue, ~37%), and cluster 3 (red, ~13%).

Analogously, the MD simulation of the FLAG peptide (DYKDDDDK) peptide also shows three different conformations as the DYKD peptide when aligned with respect to residues 1 to 4 (DYKD): (1) the semiextended (linear) one forms an S-like shape including the DYKD motif; then at D5, the peptide is bent forming a turn toward the C-terminus with a population of ~52% ($\sim 0.8 \pm 0.3$ Å all ligand atoms rmsd with respect to the minimized initial structure backbone atoms); (2) the other one forms a clamp-like shape including the DYKDD motif; then the peptide is turn toward the C-terminal with a population of ~28% ($\sim 1.8 \pm 0.2$ Å all ligand atoms rmsd with respect to minimized initial structure backbone atoms); and (3) the remaining one folds into a helix secondary structure which is better defined toward the C-terminus with a population of 20% ($\sim 2.4 \pm 0.3$ Å all ligand atoms rmsd with respect to the minimized initial structure backbone atoms) (see Figure 8). The all-residues rmsd of the FLAG peptide, as aligned with respect to all residues 1–8 (DYKDDDDK), is on an average $\sim 3.5 \pm 0.8$ Å, showing the peptide's high flexibility (see Figure S14a, Supporting Information). As the alignment is done with respect to the N-terminus residues 1–4 (DYKD), the average rmsd is reduced to $\sim 1.4 \pm 0.7$ Å, showing less flexibility of these four residues which are the ones to recognize the MIP template made by DYKDC peptide (see Figure 8a). In the

remaining portion of FLAG, the C-terminus residues 5–8 (DDDK) also show a lower average rmsd of $\sim 1.6 \pm 0.4$ Å (see Figure S14b, Supporting Information). The two portions of the peptide aligned well with respect to themselves, but not overall. They seem to move independently from each other. All rmsds were calculated with respect to the minimized initial structure backbone atoms.

Figure 8. Computational study of DYKDDDDK (FLAG) peptide in water. (a) rmsd of the FLAG peptide with respect to minimized initial structure backbone atoms aligned with respect to residues 1–4 (DYKD). (b) Left panel: two-dimensional rmsd including residues 1–4 (DYKD); right panel: cluster analysis: the three main representative conformations are depicted – cluster 1 (orange, ~52%), cluster 2 (blue, ~28%) and cluster 3 (red, ~20%).

All in all, the DYKDDDDK (FLAG) and DYKD peptides share the same number of conformations (i.e., 3) and the same most populated conformation, which is similar to one of the conformations exhibited by the template molecule DYKDC peptide used to make the MIP. However, the conformations of the FLAG peptide interconvert back and forth from one kind to another opposite to what happens to the DYKD peptide conformations, which occur more like in blocks, along the MD simulation (See Figures 7 and 8). This behavior of the FLAG peptide, favored by its longer extension of 8 residues and hence its flexibility, could explain why the DYKDDDDK (FLAG) peptide compared to the DYKD peptide recognizes better the MIP formed by the template DYKDC peptide. The quick conformational changes of DYKDDDDK (FLAG) peptide could localize and fit into the MIP surface faster than the

DYKD, thus displaying a higher selectivity toward the aforementioned hierarchically MIPs.

CONCLUSIONS

We have demonstrated that it is possible to circumvent one of the major drawbacks for the preparation of peptide-selective MIPs by an optimization of the polymer synthesis from the very first step: the grafting of the silane moieties. A rational choice of the reaction conditions leads to a well-distributed silane unit onto the silica surface. This fact has been faithfully proved in this comprehensive study by the synthesis of an epitope hierarchically imprinted polymer for the recognition of the FLAG tag by an initial immobilization of the pentapeptide DYKDC in porous silica beads. The optimized hierarchically EAMA-based imprinted polymer (MIP-AZA) shows unprecedented selectivity for FLAG peptide recognition, highlighting the importance of properly selecting the silane coupling agent and the template molecule orientation. Computational molecular modeling has also shown to be a suitable tool to explain the molecular recognition properties of the imprinted material for the FLAG peptide. Moreover, it could be of help for the selection of epitopes for protein imprinting. The developed imprinted material can be considered as an excellent candidate for future universal successful FLAG-tagged protein purification.

EXPERIMENTAL SECTION

Rehydroxylation of silica beads. Silica beads (20 g, 40–75 μm diameter, 500 Å pore size, SiliCycle) were suspended in 120 mL of 20% HCl (v/v) in a 500 mL round-bottomed flask and heated at reflux at 110 °C for 6 h. Then, the silica was filtered through a filter funnel and washed with water until pH of washing solution was around 7. Finally, the silica was dried in a vacuum oven at 70 °C for 24 h.

Optimization of derivatization process using AEAPMS.

Concentration optimization. Rehydroxylated silica beads (1 g) were suspended in 20 mL dry toluene in a three-necked round-bottomed flask connected to an automatic rotator system equipped with an overhead paddle stirring rod. This system was connected to a Dean-Stark collector coupled to a refrigerator. The mixture was then heated at 50 °C for 30 min. The appropriate amount of AEAPMS (3.75, 7.5, or 11.25 mmol) was added to the suspended beads together with PTSA (0.04 mmol). The products were designated as Si-DMPTSA1, Si-DMPTSA2 and Si-DMPTSA3 in ascending order of AEAPMS amount in the feed. The temperature was then raised to 110 °C and maintained for 2 h. The product was poured through a filter funnel and washed with 50 mL of toluene and 3x50 mL aliquots of ethanol. The functionalized silica particles were first dried in an oven at 100 °C for 24 h and finally dried in a vacuum oven at 45 °C for 24 h.

Temperature optimization. Rehydroxylated silica beads (1 g) were suspended in 20 mL dry toluene and heated at 50 °C for 30 min. Silane AEAPMS (3.75 mmol) together with PTSA (0.04 mmol) were added to the reaction mixture. The temperature was then raised to 110 °C and maintained 16 h. The product was designated as Si-DMPTSA4. Downstream procedure is analogous as described previously.

For comparison purposes, the rehydroxylated silica beads (1 g) were suspended in 20 mL dry toluene and stirred in an orbital shaker (KS 4000i, IKA) for 30 min at room temperature. Then, the silane AEAPMS (3.75 mmol) was added to the reaction and the mixture was continuously stirred for 24 h under an inert atmosphere. The product was filtered through a filter funnel and washed with 50 mL of toluene and 3x50 mL aliquots of ethanol. The functionalized silica particles were first dried in an oven at 100 °C for 16 h and finally dried in a vacuum oven at 45 °C for 24 h. Obtained product was designated as Si-DMRT.

Optimization of derivatization process using AETAZS.

Rehydroxylated silica beads (2 g) were purged under a nitrogen stream for 5 min, suspended in 20 mL dry toluene at room temperature and additionally purged for 5 min. The appropriate amount of AETAZS (3.75, 7.5, or 11.25 mmol per gram of silica) was added to the suspended beads under an inert atmosphere in an orbital shaker and the mixture was continuously stirred for 24 h. The products were designated as Si-AZA1, Si-AZA2, and Si-AZA3 in ascending order of AETAZS amount in the feed. The products were filtered through a filter funnel and washed with 20 mL of toluene and 3x50 mL aliquots of ethanol. The functionalized silica particles were first dried in an oven at 70 °C for 24 h and then dried in a vacuum oven at 45 °C for 24 h.

Iodoacetylation of silica particles. Dry dimethylformamide (DMF, 3 mL), iodoacetic anhydride (0.18 mmol) and 4-dimethylaminopyridine (DMAP, 0.18 mmol) were introduced into a 5 mL vial protected from light in the case of AEAPMS-silica. Dry DMF (3 mL), iodoacetic anhydride (0.09 mmol) and DMAP (0.09 mmol) were introduced into a 5 mL vial protected from light in the case of AETAZS-silica. After 15 min of stirring, 500 mg of aminosilica particles were added to the corresponding mixture (Si-DMPTSA1 or Si-AZA1). Products were designated as Si-DM-IAC and Si-AZA-IAC, respectively. The reaction mixtures were shaken at room temperature for 72 h in an orbital shaker. Then, the obtained products were filtered through a filter funnel and washed with 3x100 mL aliquots of DMF. Finally, particles were first dried in an oven at 45 °C for 72 h and then dried in a vacuum oven for 48 h.

Peptide DYKDC coupling to iodoacetylated silica particles.

Dry DMF (4 mL), peptide DYKDC (0.04 mmol), and triethylamine (350 μL) were introduced into a 5 mL vial protected from light in the case of the dimethoxysilane route. Dry DMF (4 mL), peptide DYKDC (0.02 mmol), and triethylamine (175 μL) were introduced into a 5 mL vial in the case of the azasilane route. After 15 min of stirring, 500 mg of iodoacetylated silica particles were added to the corresponding mixture (Si-DM-IAC or Si-AZA-IAC). The reaction mixtures were shaken at room temperature for 72 h in an orbital shaker. Then, the obtained products were filtered through a filter funnel and washed with 2x100 mL aliquots of DMF. Finally, the particles were first dried in an oven at 45 °C for 72 h and then dried in a vacuum oven for 48 h. The obtained products were designated as Si-DM-Pept and Si-AZA-Pept.

Polymer synthesis by hierarchical imprinting. The MIPs were synthesized using the corresponding peptide-derivatized silica particles as template and scaffold for polymerization (Si-DM-Pept or Si-AZA-Pept). NIPs were prepared with the corresponding preceding iodoacetylated silica particles (Si-DM-

IAC or Si-AZA-IAC). MIPs and NIPs were synthesized under the same experimental conditions. For polymerization, silica particles were packed in solid-phase extraction cartridges (5 mL, ICO plus3) equipped with a bottom SPE frit (20 μ m pore size, Agilent). The cartridge was then sealed with a rubber septum and purged under a nitrogen stream. Prepolymerization mixture was prepared in a 1 mL vial, taking into consideration the pore volume of the corresponding silica. The mixture was composed by a solution of EAMA as functional monomer, EDMA as cross-linker, DMSO as porogen and 2,2-azobis(2,4-dimethylvaleronitrile) as initiator (see Table S2 in Supporting Information for a detailed polymer composition). After purging the mixture under a nitrogen flow, the cocktail was injected into the corresponding cartridge. In order to force the mixture to completely fill the silica pores, a nitrogen stream was flow through the cartridges by closing the outlet of the SPE column. This process was repeated several times. The cartridges were checked to contain freely flowing silica particles to avoid bulk polymerization. Polymerization was done at 70 $^{\circ}$ C in an oven during 24 h. Final template/functional monomer/cross-linker (T/FM/CL) molar ratios were 0.6:4:20 for MIP-DM (using Si-DM-Pept as support) and 0.4:4:24 for MIP-AZA (using Si-AZA-Pept). After polymerization, silica beads were dissolved by adding 3 x 250 mL of 3 M aqueous solution of ammonium hydrogen difluoride and shaking the mixture for 24 h after each addition. The obtained polymers were thoroughly washed with water (until pH $\sim$ 7), 3 x 100 mL of MeOH/TFA (1%, v/v) and, finally, 3 x 100 mL of MeOH. The resulting polymer beads were dried in a vacuum oven at 50 $^{\circ}$ C for 24 h prior characterization and use. Before packing in SPE cartridges, the polymers were allowed to settle in methanol/water (80:20, v/v) to remove fine particles. NIPs were prepared under the same experimental conditions.

MISPE procedure for peptide recognition. SPE cartridges (1 mL, Varian, Palo Alto, CA, USA) were packed with 25 mg of hierarchically imprinted MIP or the corresponding NIP. The cartridges were equilibrated with 10 mL of MeOH and 10 mL of HEPES buffer (100 mM, pH 7.5). The peptide solution, containing 1 mL of DYKD and FLAG at 5 mg L $^{-1}$ was percolated at a constant flow rate of 0.4 mL min $^{-1}$ with the aid of an 8-channel Ismatec ISM936D peristaltic pump (Oak Harbor, WA, US). Then, the cartridges were rinsed with 0, 1 or 3 mL of water to remove nonspecifically retained compounds and the peptides were eluted with 1 mL water/TBA 1% (w/v). The eluates from the MISPE column were injected into the HPLC system for analysis. Aza-polymers (MIP-AZA and NIP-AZA) were subjected to a further MISPE procedure at different concentrations levels (1, 10 and 25 mg L $^{-1}$) of DYKD and FLAG peptides in order to evaluate their retention performance. The cartridges were reused at least 25 times without losing their binding properties.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsami.0c14846>. Experimental section including information about reagents and chemicals, details of the techniques used for particle characterization, HPLC methods, synthesis of hierarchically imprinted polymers, MISPE procedure for cross-reactivity studies and methods and models of computational study and Results and Discussion section including equations, 29 Si CP/MAS NMR spectra of amino-modified silica particles, characterization of derivatized silica

beads, SEM images of hierarchically imprinted polymers, and results of the computational study of DYKDC peptide/EAMA complex, and DYKD and FLAG peptides.

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