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Dual Surrogate Imprinting: An Innovative Strategy for the Preparation of Virus-Selective Particles

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Synthesis and optimization of dummy particle-imprinted polymers (HI-MIPs)

In this study, several variables affecting the formation of hemispherical indentations on the HI-MIPs surface using dummy-virus particles during the synthesis have been investigated and optimized. Two main steps can be distinguished in the synthesis process: 1) heterocoagulation and 2) polymerization. The heterocoagulation process, adapted from Ortac et al. [27], served as the initial step to link PS-NH_2 microspheres and PS-COOH nanoparticles (binding between them is achieved due to their opposite charges), while subsequent polymerization steps involved the formation of selective binding sites for the target virus on the surface of the microspheres. Within the polymerization step, further differentiation is made between the first imprinting, which involved the imprinting of dummy particles creating HI, and the second imprinting, which was based on the imprinting of the peptide. Firstly, a layer of TEOS was generated on the surface of PS-

NH_2 through sol-gel polymerization. For that, 1 µL of TEOS was added to the mixture obtained after the heterocoagulation procedure and then shaken for 24 h at 900 rpm. To evaluate this first imprinting, PS-COOH particles were removed after polymerization (24 h), aiming to leave the silica layer (TEOS) formed with the created HI. The removal of PS-COOH nanoparticles was carried out by dissolving the PS, for which different organic solvents were tested, including acetone, ethyl acetate, DMF, DMSO, and toluene (Figure S1). Among these solvents, DMF was able to dissolve the PS, leaving behind spherical core particles formed by a silica shell. This indicates that DMF effectively removed the PS while preserving the structure of the silica layer formed on the PS-NH_2 microspheres. SEM images were acquired before and after DMF washing step (Figure 1). In Figure 1A, it can be observed that PS-COOH nanoparticles were fixed to PS-NH_2 microspheres thanks to the polymerized TEOS layer around them. On the other hand, after DMF washing, the resulting morphology revealed the successful dissolving of PS and therefore the formation of well-defined HI in the silica layer (Figure 1B).

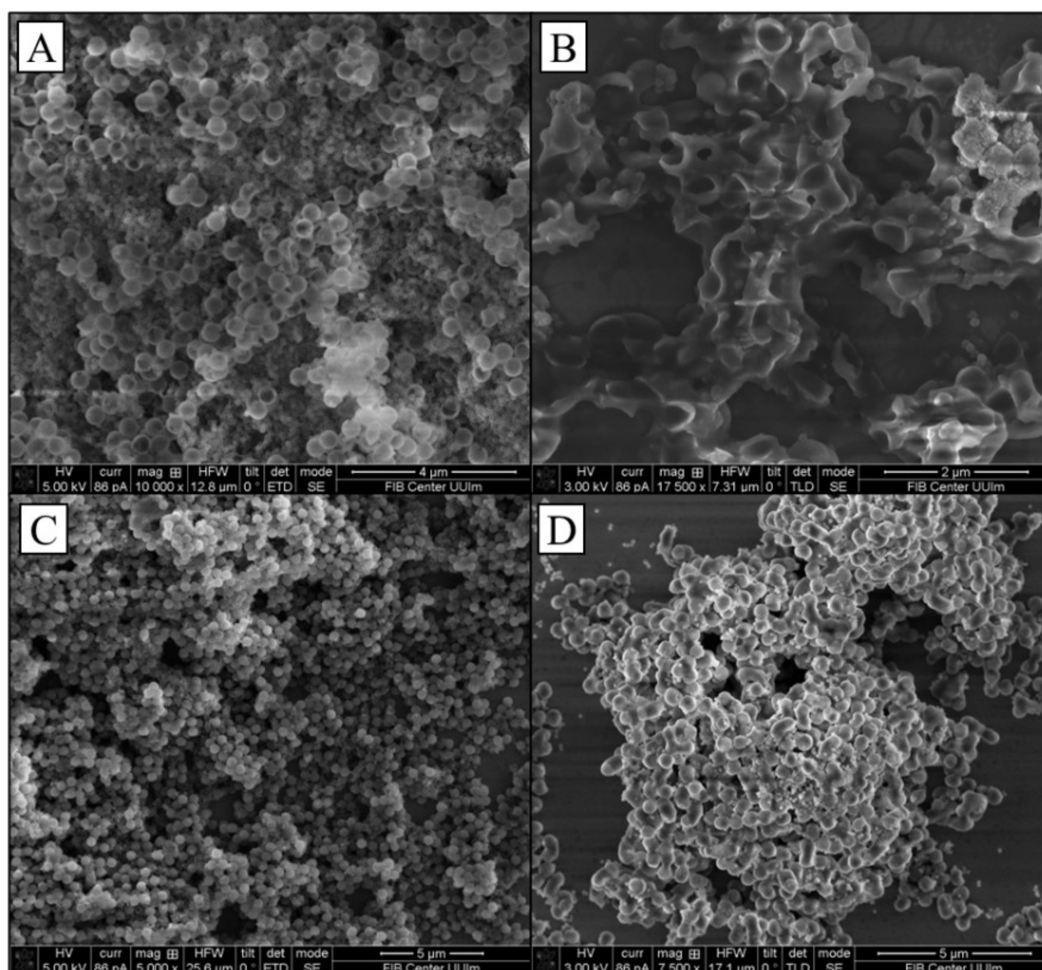

Figure S1. SEM micrographs of the washed HI-MIPs using **A)** ethyl acetate, **B)** toluene, **C)** DMSO and, **D)** acetone.

Once the optimal washing solvent for completely removing the PS particles was selected, the effect of adding higher volumes of TEOS as well as of including a co-monomer (PTES) and increasing the pH (by adding ammonium hydroxide) was studied. HI-MIPs with different volumes of TEOS (1, 1.5, 2.5, 5, and 10 μL) were prepared without the addition of ammonium hydroxide (Table S1). As shown in Figure S2 (A, B, C, D and E), in all cases it resulted in brittle HI-MIPs with an uncontrolled formation of silica over PS-COOH nanoparticles being more evident with higher TEOs amount (5 and 10 μL). This occurred because the polymerization did not occur only on the surface of the PS-NH_2 (which was the objective). Thus, in order to control the polymer formation on the surface of the PS-NH_2 and increase the thickness of the TEOS layer on them (to obtain more rigid and stable silica particles), the addition of different volumes of ammonium hydroxide (2, 5, 7.5, 10 and 15 μL) was tested (Table S1). Figure S2 (F, G, H and I) shows that increasing the pH favored the polymerization to occur on the surface of the PS-NH_2 giving rise to the formation of stable and unbreakable HI-MIPs. In this context, 7.5 μL was sufficient to control the polymerization when adding 2.5 μL of TEOS (Figure S2I). However, as shown in Figure S3, when PTES was added to the polymerization mixture as a co-monomer, 15 μL of ammonium hydroxide were needed to prepare HI-MIPs. STEM-EDAX characterization was carried out (Figure S4) to identify the silica polymeric layer created on the surface of the PS-NH_2 microspheres. EDAX analysis confirmed the presence of silica polymer around the PS core. The yellow color showed the Si regions where silica polymer was found, while the red color represented the C regions in the HI-MIPs (a predominant element in the PS). A silica polymer thickness of approx. 30-32 nm was obtained for HI-MIP-9 and HI-MIP-11.

Table S1. List of prepared and characterized HI-MIPs.

Label	TEOS (µL)	PTES (µL)	NH_4OH (µL)
HI-MIP-1	1	0	0
HI-MIP-2	1.5	0	0
HI-MIP-3	2.5	0	0
HI-MIP-4	5	0	0
HI-MIP-5	10	0	0
HI-MIP-6	1	0	2
HI-MIP-7	1.5	0	2
HI-MIP-8	1.5	0	5
HI-MIP-9	2.5	0	7.5
HI-MIP-10	1.5	0.5	10
HI-MIP-11	1.5	0.5	15

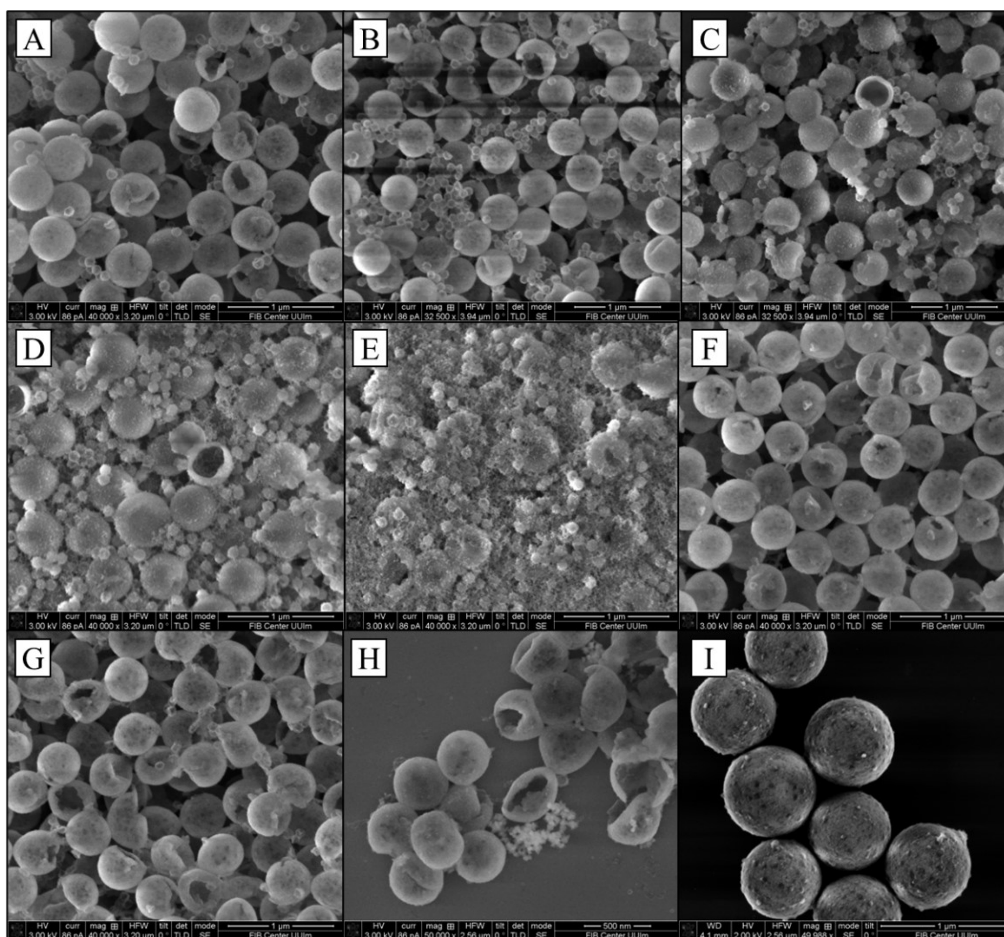

Figure S2. SEM micrographs of **A)** HI-MIP-1**, B)** HI-MIP-2, **C)** HI-MIP-3, **D)** HI-MIP-4, **E)** HI-MIP-5, **F)** HI-MIP-6, **G)** HI-MIP-7, **H)** HI-MIP-8, and **I)** HI-MIP-9.

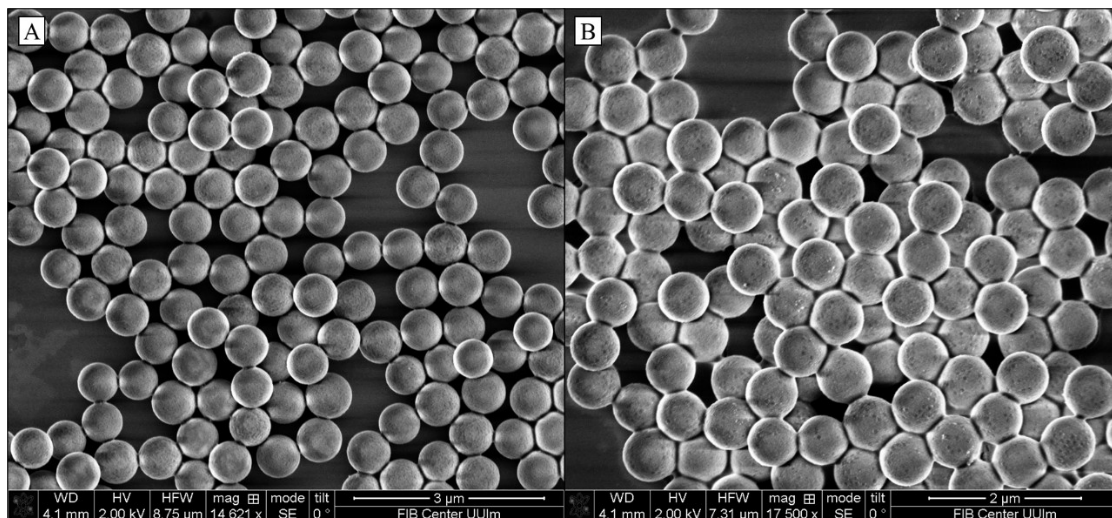

Figure S3. SEM micrographs of **A)** HI-MIP-10, and **B)** HI-MIP-11.

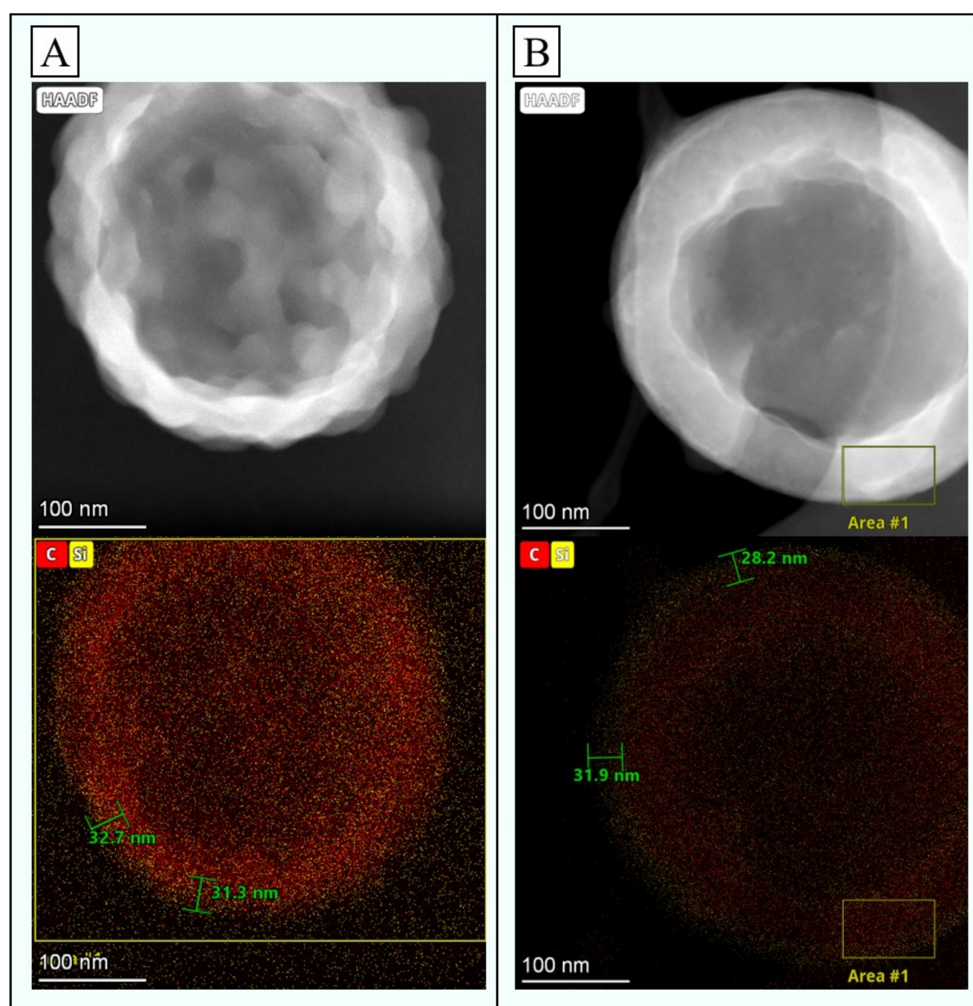

Figure S4. STEM/EDAX of **A)** HI-MIP-9 and **B)** HI-MIP-11.

Synthesis of the dual surrogate-imprinted polymers (D-MIPs) adding the peptide at 2 h after the polymerization initiation

In this approach, the polymerization reaction started without peptide addition. After 2 h, the SARS-CoV-2 peptide (107.5 µL) and an additional 1.5 µL of TEOS were added to the mixture, which was then polymerized for 24 h shaking at 900 rpm. Next, the obtained D-MIPs were washed with DMF, DMSO, and Milli-Q water. Centrifugation was used for the separation of the D-MIPs from the solvents. HI-MIPs were prepared following the same procedure but without adding the peptide (second template). The obtained D-MIPs and HI-MIPs, listed in Table S2, were observed under SEM (Figure S5A and B) to visually confirm the formation of the HI. The incorporation of the peptide at 2 h from the start of polymerization allowed the formation of the HI.

Table S2. List of H-MIPs and D-MIPs prepared adding the peptide at 2 h after the polymerization initiation.

Label	t=0		t=2h		
	TEOS (µL)	NH4OH (µL)	TEOS (µL)	PTES (µL)	SARS-CoV-2 peptide (µL)
HI-MIP-12	2.5	7.5	1.5	0	0
D-MIP-12	2.5	7.5	1.5	0	107.5
HI-MIP-13	2.5	7.5	1	0.5	0
D-MIP-13	2.5	7.5	1	0.5	107.5

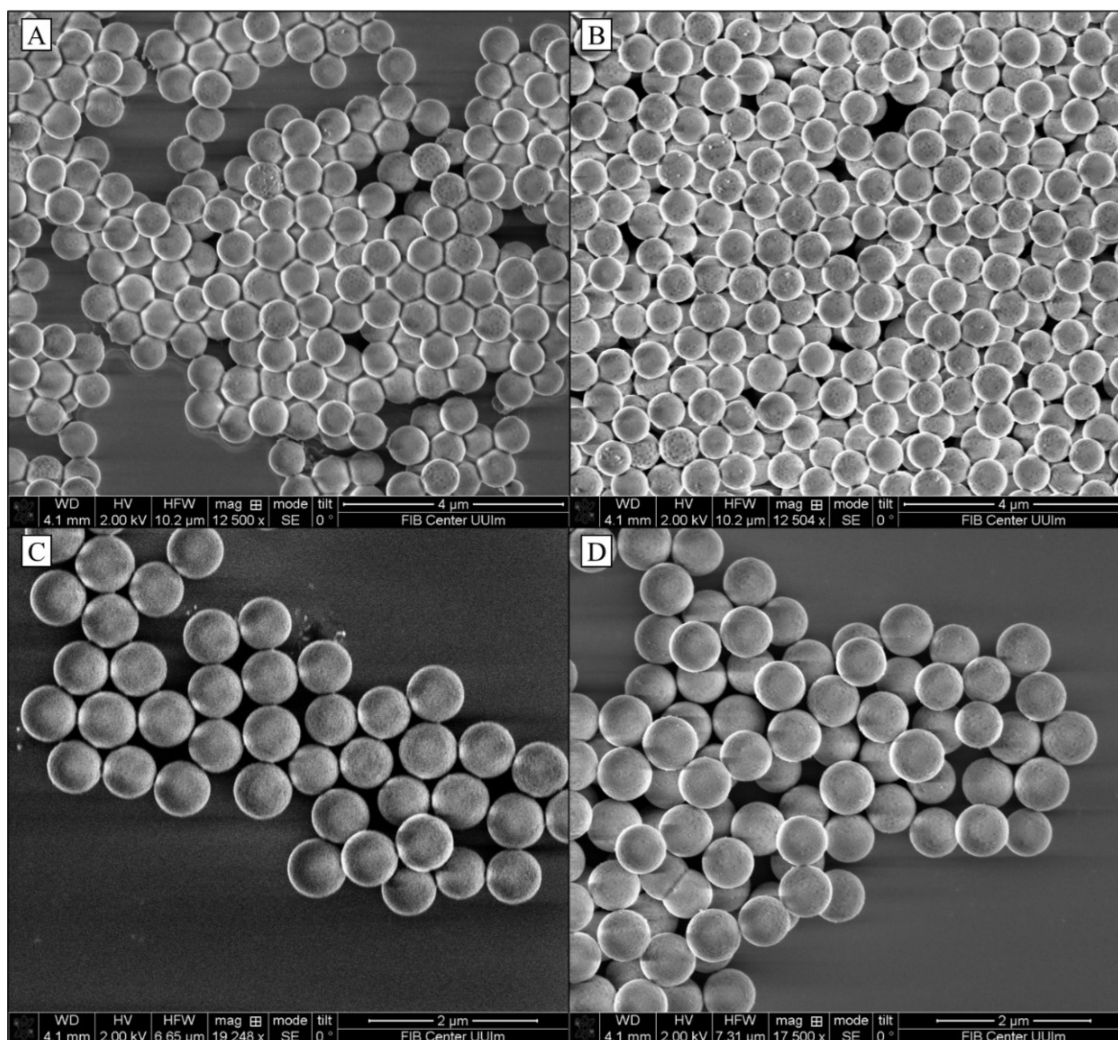

Figure S5. SEM micrographs of A) HI-MIP-12, B) D-MIP-12, C) HI-MIP-13, and D) D-MIP-13.

To confirm the creation of selective binding sites for the SARS-CoV-2 peptide, a rebinding study was conducted. For this purpose, the obtained D-MIPs and HI-MIPs were incubated in aqueous solutions containing the SARS-CoV-2 peptide, as described in Section 2.4. However, D-MIPs did not exhibit selectivity towards the peptide (Figure S6A). This could be attributed to the use of a single monomer (TEOS) for the preparation of D-MIPs may have limited the formation of selective binding sites for the peptide.

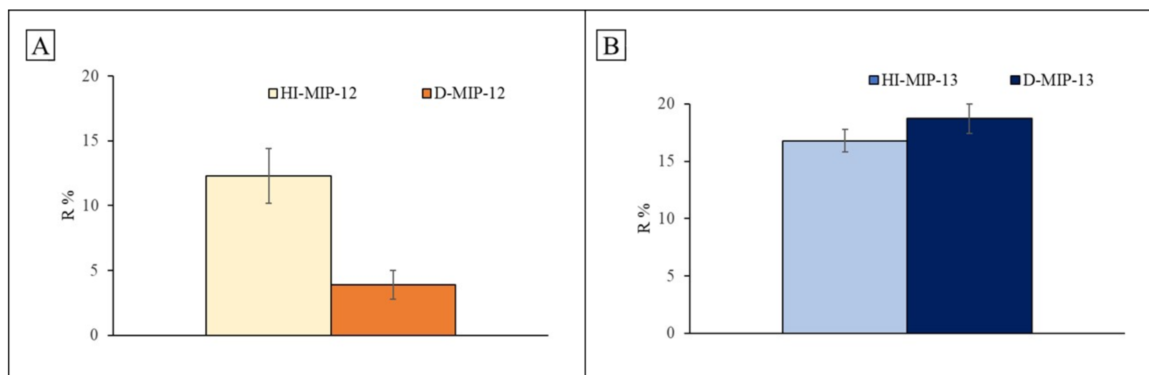

Figure S6. SARS-CoV-2 peptide binding efficiency of HI-MIPs and D-MIPs prepared via adding the peptide at 2 h using **A)** TEOS and **B)** TEOS/PTES as monomers.

D-MIPs using TEOS and PTES were also prepared following the same procedure. In this way, HI-MIP-13 and D-MIP-13 were prepared according to the Table S2. As expected, HI were created on the surface of HI-MIP-13 and D-MIP-13 (Figure S5C and D). Although the HI were more shallow than the obtained for HI-MIP-12 and D-MIP-12, it demonstrated that the late addition of the peptide allowed the polymerization to start on the surface of the PS-NH_2 allowing the imprinting of the dummy-virus particles. Moreover, the inclusion of a functional co-monomer (PTES) led to a slight improvement in the selectivity of the D-MIPs for recognizing the SARS-CoV-2 peptide (Figure S6B) compared to the rebinding results of the polymers prepared using only TEOS (Figure S6A).

Synthesis of the dual surrogate-imprinted polymers (D-MIPs) adding the peptide at 4 h after the polymerization initiation (three-step polymerization method)

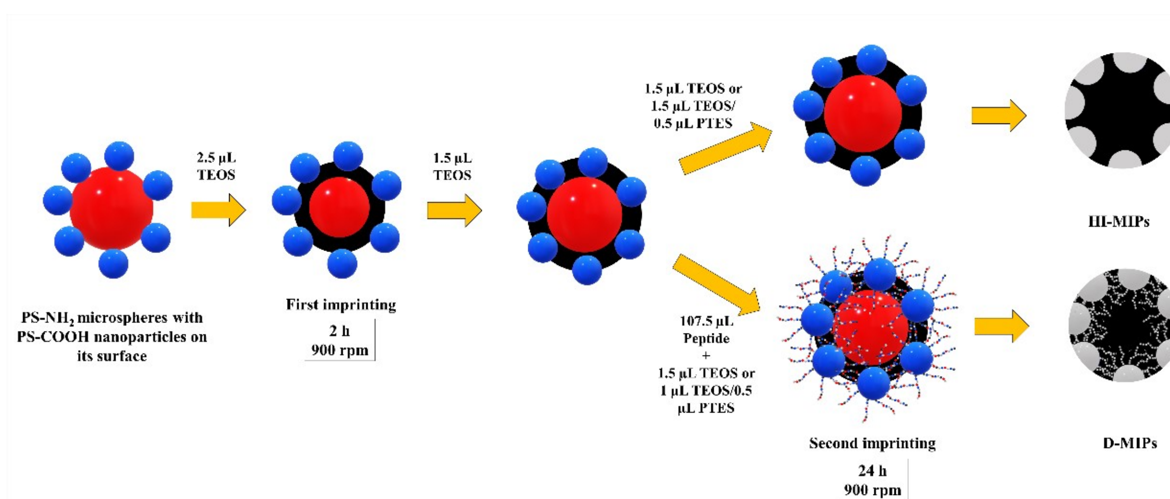

Figure S7. General scheme of the three-step polymerization method used to synthesize DMIPs and HI-MIPs.

Table S3. List of H-MIPs and D-MIPs prepared adding the peptide at 4 h after the polymerization initiation.

Label	t=0		t=2h		t=4h		
	TEOS (µL)	NH4OH (µL)	TEOS (µL)	PTES (µL)	TEOS (µL)	PTES (µL)	SARS-CoV-2 peptide (µL)
HI-MIP-14	2.5	7.5	1.5	0	1.5	0	0
D-MIP-14	2.5	7.5	1.5	0	1.5	0	107.5
HI-MIP-15	2.5	7.5	1.5	0	1	0.5	0
D-MIP-15	2.5	7.5	1.5	0	1	0.5	107.5

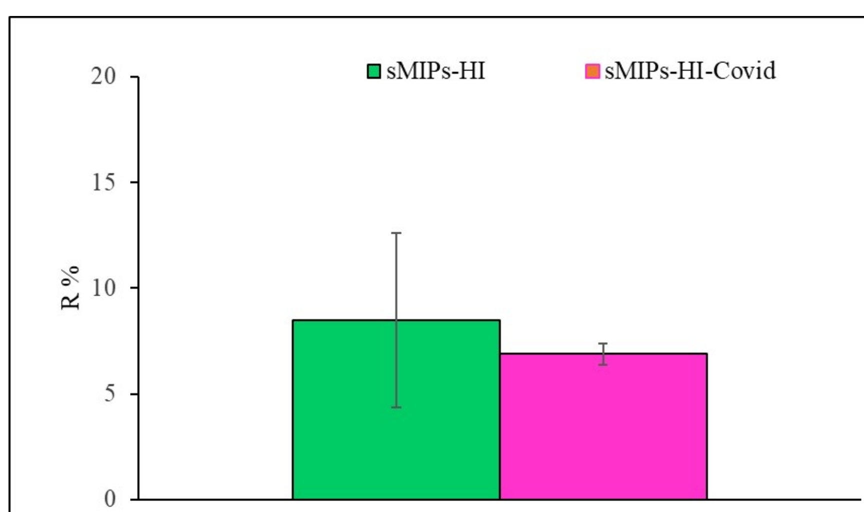

Figure S8. Zika peptide binding efficiency of HI-MIP-15 and D-MIP-15.

Instrumentation

The incubation of the PS-NH_2 microspheres and PS-COOH nanoparticles as well as the subsequent polymerization step was performed in a Thermomixer C (Eppendorf, Germany). A HERAEUS Megafuge 16 centrifuge from Thermo Scientific was used for the separation of the PS particles and final MIPs.

For the characterization of the surrogate-imprinted polymers, a Helios Nanolab 600 (scanning electron microscope, SEM) was used for characterizing the resulting polymers. They were dispersed in water and a drop was deposited in the SEM support. Once it had been dried, the SEM analyses were carried out. Moreover, Talos F200i Transmission

Electron Microscope (TEM) with STEM-HAADF transmission scanning imaging system, energy dispersive X-ray microanalysis system, EDX and digital camera.
For SARS-CoV-2 characterization, negative staining-TEM (JEOL 1400 transmission electron microscope) was performed as previously described (J Am Chem Soc. 2 (2022) 2187-2202). In brief, 10 µL of the colloid suspension was loaded onto freshly glow-discharged 300 mesh copper grids coated with a carbon-reinforced formvar film. After 10 min of adsorption at room temperature, grids were washed three times with aqua bidest and stained with 2% uranyl acetate in aqua bidest. Grids were imaged and operated at 120 kV.

Cell culture and viruses
All cells were cultured at 37°C in a 5% CO2 and 90% humidity. Human embryonic kidney (HEK) 293T and Vero E6 cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (PANBiotech), 100 µg/ml streptomycin (PANBiotech) and 100 U/ml penicillin (PANBiotech). Caco-2 (human epithelial colorectal adenocarcinoma, kindly provided by Prof. Holger Barth, Ulm University) cells were cultivated in DMEM (Gibco) containing 10% FBS (Gibco), 2 mM glutamine (PANBiotech), 100 µg/ml streptomycin (PANBiotech), 100 U/ml penicillin (PANBiotech), 1 mM Non-essential amino acids (NEAA, Gibco), 1 mM sodium pyruvate (Gibco).

Viruses
The SARS-CoV-2 variant B.1.617.2 (Delta) was kindly provided by Prof. Dr. Florian Schmidt and Dr. Bianca Schulte (University of Bonn). To propagate the virus, 70-90% confluent Vero E6 cells, seeded in 10 cm cell culture dishes were inoculated with the SARS-CoV-2 Delta isolate (MOI of 0.03) in 3.5 ml serum-free medium. The cells were incubated for 2h at 37°C, before adding 20 ml medium containing 15 mM HEPES (Carl Roth, Cat#6763.1). Virus stocks were harvested as soon as strong cytopathic effect (CPE) became apparent, typically after about 12 hrs. The virus stocks were centrifuged for 5 min at 1,000 g to remove cellular debris, aliquoted, and stored at -80°C until further use.

Tissue Culture Infection Dose50 (TCID50) endpoint titration

SARS-CoV-2 stocks or infectious supernatants were serially diluted on Caco-2 (15,000), seeded in 96 F-bottom plates in 100 µl medium and incubated overnight. 100 µl of diluted SARS-CoV-2 stocks or infectious supernatants were used for infection, resulting in final dilutions of 1:10^1 to 1:10^12 on the cells in 8 technical replicates. Cells were incubated for 7 days and monitored for CPE. TCID50/ml was calculated according to the Reed and Muench method.

Statistics

Statistical analyses were performed using GraphPad PRISM 9.2 (GraphPad Software). P-values were determined using a One-Way ANOVA. Unless otherwise stated, data are shown as the mean of at least three independent experiments ± SEM.