

Peptide-Functionalized Gold Nanoparticles Targeting PD-L1: Design via a Molecular Dynamic Driven Approach and Further Experimental Validation

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Cite This: *ACS Appl. Bio Mater.* 2026, 9, 7637–7649



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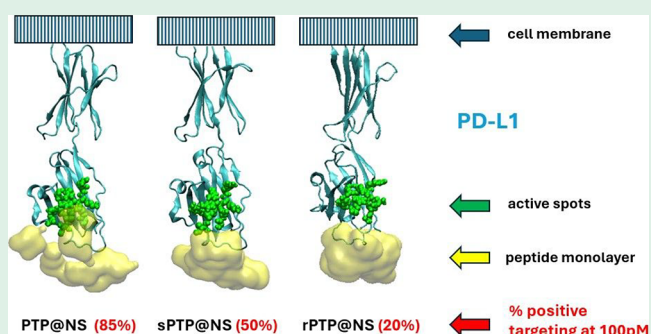
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ABSTRACT: The development of theranostic tools for the early detection and localization of tumors represents a major challenge in oncology. Among emerging strategies, the targeting of Programmed Death Ligand-1 (PD-L1), a key immune checkpoint protein overexpressed in many tumor types, has gained significant attention. In this work, we report design and development of theranostic gold nanostructures functionalized with PD-L1-targeting peptides (PTP, sPTP, and rPTP) whose sequences were identified combining structural analysis of the PD-1/PD-L1 interaction interface and molecular dynamics simulations. This is because the design of functional nanostructures for protein targeting requires a precise understanding of how molecular recognition is affected by ligand organization at interfaces; therefore, peptide design was guided not only by the selection of key residues involved in binding but also by the evaluation of peptide assemblies to explicitly account for the collective effects governing target recognition. Indeed, beyond conventional evaluation of protein/single-peptide interaction, peptide clusters and surface-anchored monolayers were investigated to consider features like peptide assembly, organization, and reduced conformational freedom in the nanostructure/PD-L1 interaction. Results indicate that peptide sequence and orientation critically determine monolayer organization and accessibility of the PD-L1 binding motif. The computational predictions were experimentally validated by synthesizing peptide-functionalized gold nanostructures and evaluating their targeting performance against MDA-MB-231 breast cancer cells over-expressing PD-L1, using the surface-enhanced Raman scattering technique: NS functionalized with PTPs achieved the targeting of approximately 85% of MDA-MB-231 cells at 100 pM nanostructure concentration, compared to 23% for those functionalized with rPTP, demonstrating a nearly four-fold difference attributable exclusively to peptide orientation on the nanostructure surface. The specific system investigated in this work establishes a computational framework for the rational design of peptide-functionalized nanostructures, providing insights into the collective behavior of peptide monolayers and offering a smart methodology that, while demonstrated here for targeting PD-L1, is in principle applicable to other protein targets.

KEYWORDS: peptide-functionalized nanoparticle, PD-L1, SERS, gold nanoparticle, nanostructures, molecular dynamic simulations



INTRODUCTION

The rapid advancement of nanotechnology is reshaping the biomedical landscape, enabling innovative strategies for the diagnosis, treatment, and prevention of human diseases.^{1,2} Among the nanomaterials developed for biomedical applications, gold nanoparticles (AuNPs) have emerged as one of the most versatile and extensively investigated systems. Their success arises from their unique combination of physicochemical properties, including a tunable optical response governed by localized surface plasmon resonance (LSPR), high biocompatibility, chemical stability in biological environments, and readily engineered surface chemistry. These features have established AuNPs as a

robust and adaptable platform in a wide range of biomedical applications.^{3–5}

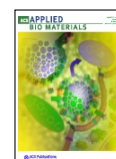
In biomedicine, peptide- and antibody-functionalized AuNPs have demonstrated great potential and broad applicability. They have been indeed explored for example, as antimicrobial

Received: May 26, 2026

Revised: July 22, 2026

Accepted: July 24, 2026

Published: August 3, 2026



agents,^{6,7} as carriers for nucleic acid delivery, as contrast agents for multimodal imaging, and as platforms for biosensing.^{8–10}

In this context, cancer theranostic has emerged as one of the most promising applications of AuNPs. Their small size and surface properties enable passive accumulation at tumor sites through the enhanced permeation and retention effect, while active targeting can be achieved by functionalizing the nanoparticle surface with ligands selective for tumor-associated molecular markers. From a diagnostic standpoint, the plasmonic properties of AuNPs underpin surface-enhanced Raman scattering (SERS), in which intense electromagnetic field enhancement at nanoscale hot spots amplifies the Raman signals of reporter molecules by several orders of magnitude, allowing sensitive and multiplexed detection of tumor cells with a spectral specificity that fluorescence-based methods cannot match.^{11,12} From a therapeutic point of view, the strong near-infrared absorption of gold nanostructures can be exploited for photothermal therapy, allowing localized heat generation upon laser irradiation, which selectively destroys tumor cells while minimizing damage to surrounding healthy tissues.^{13,14} The integration of these capabilities into a single nanoplatform defines the theranostic paradigm that has made AuNPs one of the most actively pursued systems in precision oncology.^{15,16} In this framework, the development of AuNP-based functionalized nanostructures (NSs) to target the immune checkpoint proteins PD-1 or PD-L1 is a promising strategy in the field of cancer immunotherapy.^{17–21} PD-1, an inhibitory receptor expressed on activated T cells, interacts with its primary ligand, PD-L1,^{22,23} which is frequently overexpressed on the surface of many tumor cells, including non-small cell lung cancer, melanoma, triple-negative breast cancer, renal cell carcinoma, and glioblastoma cells. The interaction between PD-1 and PD-L1 delivers an inhibitory signal to the T cell, leading to a state of T cell exhaustion, making them ineffective in implementing a robust antitumor response and allowing cancer cells to evade immune surveillance and proliferate unchecked.^{24,25} To this end, the structural and energetic determinants of the PD-1/PD-L1 interaction have been extensively investigated through computational approaches, including molecular dynamics (MD) simulations, free-energy calculations, and structure-based design strategies, providing detailed information on the binding interface and key residues that govern complex stability.^{26–29} Based on these studies, in our previous research, we have developed NSs constituted by AuNPs functionalized with chemisorbed peptides capable of targeting PD-L1. The compelling need for the design of ad hoc nanostructures for the targeting of PD-1 or PD-L1 is justified by several considerations. First, the use of monoclonal antibodies, even if it has led to fundamental advantages in cancer therapy, targeting PD-1 and PD-L1 due to their high affinity, prolonged circulation time, and proven therapeutic efficacy; nevertheless, it has some limitations, such as high production costs, the potential for infusion reactions, and the risk of generating antidrug antibodies, which can decrease therapeutic efficacy or trigger an adverse immune response.^{30–35} Valid emerging therapeutic alternatives in cancer immunotherapy are represented by the use of tuned low-molecular-weight inhibitors such as aptamers,^{36,37} small molecules such as biaryl compounds,^{28,38,39} and peptides and peptidomimetic molecules.^{40–48} Their advantages reside in cost-effective synthesis, chemical tunability, low immunogenicity, and enhanced tissue penetrations, but at the same time, they present some challenges and limitations toward clinical

applicability. For example, aptamers are characterized by poor cellular internalization and susceptibility to nuclease digestion, which shorten the half-life and, in turn, efficacy. To reduce these drawbacks, several strategies such chemical modifications^{36,49} and nanoparticle functionalization⁵⁰ have been taken into account. As for small molecules, such as BMS,^{28,51} their large potential as inhibitors is limited by the difficulty of targeting the flat active site of the protein responsible for PD-L1/PD-1 interaction. Targeting peptides^{40–47} have the advantage of mimicking native protein–protein interaction motifs, but they still exhibit several intrinsic limitations:^{41–43,52,53} free peptides often exhibit lower receptor affinity compared to antibodies and are susceptible to proteolytic degradation, which can limit their potential.^{54–57} Therefore, to overcome these drawbacks, nanoparticles (e.g., polymeric or metal nanoparticles, liposomes, dendrimers) functionalized with peptides can be used.^{46,47,58} Functionalization of nanoparticles with peptides in the literature has been shown to serve a dual purpose: it enhances the stability of the peptides, protects them from degradation, and increases the local concentration of peptides, thus favoring significantly cooperative binding and avidity while improving affinity to the target receptor.⁵⁹ Furthermore, as explained above, the use of AuNPs has the further advantage of being exploited as SERS-active nanostructures for rapid verification of tumor cell targeting, as demonstrated in recent years.^{43,60–62} However, the building up of peptide-functionalized nanoparticles requires an optimization rational strategy. More in detail, for example, we previously developed gold nanostructures that carry by chemisorption a peptide capable of interacting with PD-L1. The peptide, called CLP002, identified by bacterial surface display methods, exhibits high affinity for the target ($K_d \approx 0.4 \mu\text{M}$).⁶³ Experimental studies carried out with gold nanostructures functionalized with CLP002 or its scrambled sequence have provided important information, highlighting the critical role of the peptide sequence, the spacer design, and the complex network of intermolecular interactions formed after surface immobilization. MD simulations proved essential in rationalizing these observations, revealing that peptide assemblies arising from interchain interactions significantly influence both binding efficiency and system stability. In fact, chemical anchoring of peptides on nanostructures enables precise control over peptide exposure to the solvent, orientation, and local density; this means that the targeting efficiency of peptide-functionalized gold nanostructures is not solely dictated by the intrinsic affinity of the peptide sequence, but is strongly influenced by its organization on the nanoparticle surface.^{60,62,64}

These findings emphasize the complexity of designing effective peptide-based nanotherapeutics and highlight a key unmet need: the development of predictive computational protocols capable of guiding the design of peptides before experimental validation, taking into account not only intrinsic affinity between peptides and the target, but also the collective behavior of peptides in their functional, surface-bound state. The peptides designed in this work differ from CLP002 in both origin and design strategy: while CLP002 was identified by bacterial surface display and functionalized through a long poly(ethylene)glycol-based spacer with charged moieties to enhance the affinity, the PTP and sPTP peptides were rationally engineered de novo analyzing the PD-1/PD-L1 interaction interface and were anchored through a short diethylene glycol spacer (O2oc-Cys; see Table 1). This architectural simplification, combined with the sequence-driven design, represents a conceptual

Table 1. Peptides with Linkers and Relative Acronyms Used in the Functionalization of Nanostructures (NSs)

peptide	sequence ^a	NS acronyms
PTP	Ac-His-Leu-Tyr-Glu-Ile-Val-Gln-His-Ile-Gly-Asn-Leu-Arg-Trp-Thr-Ile-O2oc-Cys-NH ₂	PTP@NS
sPTP	Ac-Ile-Gln-Ile-Arg-Glu-Gly-Ile-Leu-Tyr-Asn-Thr-His-Trp-Val-Leu-His-O2oc-Cys-NH ₂	sPTP@NS
rPTP	Ac-Cys-Lys3-Gly2-His-Leu-Tyr-Glu-Ile-Val-Gln-His-Ile-Gly-Asn-Leu-Arg-Trp-Thr-Ile-NH ₂	rPTP@NS

^aStandard abbreviation for natural amino acids; Ac: acetyl group; O2oc: $-\text{NH}(\text{CH}_2\text{CH}_2\text{O})_2\text{CH}_2-\text{CO}-$.

advance of the present work in relation to previous CLP002-based nanostructures.

Based on these considerations, in this work, we address this challenge by designing and synthesizing gold nanostructures functionalized with de novo engineered PD-L1 targeting peptides with enhanced affinity and theranostic potential. Our approach relies on a robust computational strategy: amino acid residues of peptides were selected based on their involvement in the PD-1/PD-L1 interaction interface, identified through MD simulations performed starting from the X-ray structure of the complex. Two peptide sequences were then designed: His-Leu-Tyr-Glu-Ile-Val-Gln-His-Ile-Gly-Asn-Leu-Arg-Trp-Thr-Ile and Ile-Gln-Ile-Arg-Glu-Gly-Ile-Leu-Tyr-Asn-Thr-His-Trp-Val-Leu-His. Although the identification of key residues involved in the PD-1/PD-L1 recognition has been previously reported,^{44,45} the novelty of our approach lies in the definition of a general protocol that enables the design of the peptide a priori by linking sequence selection, spatial organization, and expected interaction mode prior to experimental validation.

To assess peptide affinity for PD-L1, for a first screening, their interactions were first evaluated by MD simulations in aqueous solution. Importantly, the proposed strategy accounts for peptides chemisorbed on the nanoparticle surface, where they experience similar orientations, high local density with consequently reduced conformational freedom, and interchain interactions: these features lead to crowding and partial shielding of functional residues outward.

Based on these considerations, MD simulations were performed on peptide clusters composed of three parallel peptides, mimicking their arrangement on the nanoparticle surface and avoiding unrealistic conformations. This approach enables the evaluation of peptide–PD-L1 interactions under conditions that closely resemble their operational configuration. In a second step, MD simulations of PD-L1 interacting with peptide monolayers on gold surfaces were carried out, providing a more realistic representation of functionalized nanostructures. Finally, the peptides were synthesized and chemisorbed on AuNPs forming nanostructures (NSs), and the in vitro targeting of PTP@NS, sPTP@NS, and rPTP@NS (see Table 1) against MDA-MB-231 cells over-expressing PD-L1 was evaluated through SERS, showing strong agreement between experimental data and computational predictions.

This work establishes a first important step toward an experimentally validated rational design framework for peptide-functionalized gold nanostructures, explicitly considering peptide assembly, orientation, and surface organization and thus circumventing the known limitations of the use of peptides as PD-L1 targeting systems, a procedure generally not considered in conventional ligand screening approaches. This design reduces the trial-and-error components and accelerates the development of theranostic platforms. Although the framework is validated here on a single target system with three peptide

variants, the underlying protocol is designed to be generalizable across different peptide sequences and surface-accessible protein targets.

■ MATERIALS AND METHODS

Molecular Dynamics Simulations

General Protocol. All molecular dynamics (MD) simulations were performed using the GROMACS 2024 software package.⁶⁵ The GROMOS53a6 force field was used, with additional parameters that describe Au atoms. Parameters for the O2oc spacer were assigned by analogy with chemically related moieties available within the force field.

Unless otherwise specified, the following simulation protocol was applied to all systems investigated. The explicit solvent was modeled using the simple point charge (SPC) water model, and periodic boundary conditions (PBCs) were applied in all three spatial dimensions. All simulations were performed at the constant pressure and temperature ensemble (NPT). Energy minimization was first carried out using the steepest descent algorithm until convergence. The systems were subsequently equilibrated for 100 ps at 300 K using a 0.5 fs time step. Production runs were then conducted with a 2 fs time step. Long-range electrostatic interactions were treated using the particle mesh Ewald (PME) method with a real-space cut-off of 1.4 nm.^{66,67} The same cut-off (1.4 nm) was applied for van der Waals interactions. Temperature was controlled using the velocity-rescale thermostat ($\sigma_T = 0.6$ ps), while pressure was maintained under isotropic conditions using the C-rescale barostat ($\tau_p = 1$ ps).⁶⁸

The starting structures of PD-L1 and PD-1 were those of the crystal structure of the complex present in the Protein Data Bank (pdb id: 3SBW).

To avoid biases due to the initial structure, all simulated peptides (see Table 1) were set in the all-trans conformation.

The solvent-accessible surface area (SASA) was calculated using the GROMACS tool *sasa*. Interaction energies were evaluated using the GROMACS *energy* module. The number of contacts was determined using a homemade code that counts pairs of atoms with a separation of less than 0.5 nm. Structural visualization and trajectory inspection were performed using VMD⁶⁹ and UCSF Chimera.⁷⁰

Molecular Dynamics Simulations in Solution. The attractive region between PD-1 and its ligand was obtained by running two replicas of 100 ns MD simulations of the PD-1/PD-L1 adduct, starting from the X-ray structure, solvating the simulation box with water molecules, and adding ions to ensure neutrality and correct ionic strength (see Table 2).

To analyze the peptide–PD-L1 interaction, a preliminary study was carried out in solution. To mimic the proximity of PD-L1 to the monolayer, a cluster of three parallel peptides was used, in an initial all-trans conformation, planarly arranged in close contact, with inter-peptide separation comparable to that present in peptide aggregates in a fully extended conformation.

At the beginning of all of the simulations, each peptide cluster was placed at a minimum initial distance of 1 nm from the attractive region of PD-L1 (Figure S1).

Each system was solvated with explicit water molecules (SPC), and Na⁺ and Cl[−] ions were added (see Table 2) to ensure electroneutrality and reproduce physiological ionic strength ($I = 0.156$ M).

Table 2. Size of the Boxes, Number of Added H₂O Molecules and Ions in the MD Simulation

system	box (nm ³)	water	ions (Na ⁺ /Cl ⁻)
PD-1/PD-L1	10 × 10 × 15	47,361	131/126
3 PTP/PD-L1	10 × 10 × 15	47,512	131/126
3 sPTP/PD-L1	10 × 10 × 15	47,523	131/126
3 rPTP/PD-L1	11 × 11 × 23	88,919	200/204

For each peptides/PD-L1 system, 20 independent simulations of 10 ns were performed using different initial velocity distributions.

Molecular Dynamics Simulations on the Gold Surface. As already reported,⁴³ the model gold surface was constructed by placing Au atoms in a hexagonal close-packed lattice. The resulting monolayer was energy-minimized and subsequently restrained using a harmonic positional constraint (force constant = 10,000 kJ mol⁻¹ nm⁻²) to preserve surface integrity throughout the simulations.

Peptides were placed initially in all-trans conformation, with their long axis aligned to the Z-direction (perpendicular to the gold surface) assuming random positions in the XY-plane and with random rotations around the Z-axis. The sulfur atom of the terminal cysteine was placed close (0.294 nm) to the Au layer and restrained, only at the Z coordinate, using a positional constraint (force constant = 10,000 kJ mol⁻¹ nm⁻²) to mimic the S–Au bond and allow eventual translational motion.

To prevent artificial relaxation or tilting of the extracellular PD-L1 domains on the peptide monolayer, an angular positional restraint was introduced.^{43,62} This constraint was defined using four atoms approximately aligned along the Z-direction and applied with a force constant of 500 kJ mol⁻¹ rad⁻². This setup preserved the correct orientation of PD-L1 relative to the surface and ensured physiologically significant exposure of the interaction interface.

The monolayers were constructed by inserting the maximum number of peptide molecules compatible with the box dimension, as detailed in Table 3.

For each monolayer system, two independent simulations of 100 ns were performed. In simulations involving PD-L1, the monolayer surface was conceptually divided into four quadrants (Figure S2), and the protein was positioned centrally within each quadrant to sample different local peptide environments and minimize positional bias.

The simulation parameters and composition of the systems are summarized in Table 3.

Materials and Instrumentation. Unless otherwise specified, all chemicals were purchased from commercial suppliers and used without further purification. Fmoc-protected amino acids were obtained from Merck (Merck KGaA, Inc., Darmstadt, Germany). Rink Amide AM LL resin (0.35 mmol g⁻¹ loading) and reagents for solid-phase peptide synthesis were purchased from Merck (Merck KGaA Inc., Darmstadt, Germany). Rink Amide MBHA resin (0.52 mmol g⁻¹ loading) and Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-O2oc-OH) were acquired from Iris Biotech GmbH (Marktredwitz, Bavaria, Germany). Sulforhodamine 101-bis-cysteamide (TR-SH) was used as a SERS reporter and is from Merck. Bovine serum albumin (BSA) was purchased from Merck. Peptides and conjugates were analyzed by LC/MS using a 6100 Series Single Quadrupole LC/MS system (Agilent Technologies, Inc., Santa Clara, CA, USA), which is equipped with an on-line UV–vis detector (220 and 280 nm) and operated in positive electrospray ionization (ESI) mode. Separation was achieved on a Kinetex column (100 × 4.6 mm, 3.5 μm) using a binary gradient of mobile phase A (0.1% trifluoroacetic acid in water) and mobile phase B (0.1% trifluoroacetic acid in acetonitrile). Semi-preparative HPLC purification was performed on a Shimadzu LC-6A (Shimadzu Corporation, Kyoto, Japan) system equipped with dual pumps, and UV–vis spectra were recorded on a UV–vis-NIR spectrophotometer (mod. Cary 5000, Agilent Technologies, Inc., Santa Clara, CA, USA) using 0.2 or 1 cm quartz cuvettes. Dynamic light scattering (DLS) and ζ-potential measurements were performed on a Malvern

Table 3. Number of Peptides, Size of the Boxes, Number of Added H₂O Molecules and Ions in the MD Simulation

system	box (nm ³)	peptides	water	ions (Na ⁺ /Cl ⁻)
PTP@NS	10.57 × 10.23 × 20.32	78	62,126	195/190
sPTP@NS	10.55 × 10.21 × 20.30	90	60,675	195/190
rPTP@NS	10.29 × 10.19 × 23.00	73	68,402	115/335

instrument (mod. Zetasizer Nano-ZS, Malvern Analytical Ltd., Worcestershire, UK) equipped with a 633 nm He–Ne laser. Surface-enhanced Raman scattering (SERS) spectra were acquired using a micro-Raman spectrometer (mod. InVia, Renishaw plc., Wotton-under-Edge, UK) with 633 nm excitation (0.8 mW). Measurements were made with a 20× objective (1 s acquisition) or a 10× objective (10 s acquisition) for colloidal solutions.

Synthesis of Peptides and Functionalized Nanostructures. Peptides were synthesized by Fmoc-based solid-phase peptide synthesis (SPPS) using an Advanced Chemtech 348 Ω automated peptide synthesizer. Synthesis was performed on Rink amide MBHA resin (0.05 mmol scale) or Rink amide AM resin (0.03 mmol scale). The side-chain protecting groups were as follows: tert-butyl (Thr, Tyr), tert-butyloxycarbonyl (Trp), trityl (Asn, His, Cys), and 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) for Arg. Coupling reactions were carried out in DMF for 45–60 min using 4 equivalents of Fmoc-protected amino acids activated with N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)-uronium hexafluorophosphate (HBTU), N-hydroxybenzotriazole (HOBt), and N,N-diisopropylethylamine (DIPEA) in a 4:4:12 molar ratio. Fmoc deprotection was performed using 20% piperidine in DMF (15 + 5 min cycles). After cleavage of the resin and global deprotection using a TFA/H₂O/TIS/EDT cleavage cocktail (94:2.5:2.5:1, v/v/v/v), crude peptides were precipitated with cold diethyl ether, collected by centrifugation, and purified by semi-preparative HPLC. Purity and identity were confirmed by analytical HPLC and mass spectrometry (Figures S3–S5).

Gold nanoparticles (NPs) were synthesized by laser ablation of a 99.9% pure gold target in 10 μM NaCl aqueous solution. Ablation was performed using a Nd:YAG laser (mod. YG98110E, Quantel Laser by Lumibird SA, Villejust, France) (1064 nm, 6 ns pulse duration, 10 Hz repetition rate) at a fluence of 2 J cm⁻².⁷¹ Transmission electron microscopy (TEM) analysis (using TEM JEOL F200 instrument, JEOL Ltd, Tokyo, Japan) confirmed the formation of spherical nanoparticles with an average diameter of approximately 20 nm. The nanoparticles exhibited a negative ζ-potential (~ -30 mV), attributed to partial surface oxidation occurring during plasma formation in the ablation process, ensuring good colloidal stability. The nanoparticle concentration (2–5 nM) was determined by fitting the localized surface plasmon resonance (LSPR) peak centered at 522 nm using boundary element method (BEM) simulations.⁷² SERS nanostructures were prepared by adding 5 μL of a 35 μM solution of sulforhodamine 101-bis-cysteamide (TR-SH) in methanol to 2 mL of the colloidal NP solution. Aggregation was visually confirmed by a color change from red to dark violet. The excess reporter was removed by centrifugation (4000 rcf). They were incubated, to obtain functionalized peptide nanostructures (NSs), with a millimolar peptide solution for 20 min at room temperature under stirring. Covalent anchoring of peptides to the gold surface occurred via thiol–gold interactions mediated by the cysteine residue. Excess peptide was removed by centrifugation (3000 rcf). The number of peptides per nanoparticle was estimated by measuring the difference in peptide absorbance before and after functionalization. Nanostructure hydrodynamic diameter and ζ-potential were measured by dynamic light scattering, yielding average diameters of ~160 nm and ζ-potentials of approximately -20 mV. The extinction spectrum of isolated NPs displayed a sharp LSPR peak at 522 nm, consistent with ~20 nm spherical particles. Upon aggregation during SERS-NS formation, this feature broadened and red-shifted toward ~700 nm, with a shoulder near 530 nm and decreased extinction beyond 1000 nm,

consistent with plasmonic coupling in nanoparticle aggregates. The spectral fitting indicated aggregates composed of up to ~ 10 nanoparticles per structure. Based on electromagnetic simulations, the expected SERS enhancement factor ranged from 10^9 to 10^{10} .⁷² SERS spectra were dominated by reporter (TR-SH) signals, with characteristic bands at 1505 and 1649 cm^{-1} corresponding to C–N and C–C stretching modes, respectively. These bands were used as spectral markers to confirm nanostructure presence during cellular targeting experiments. Peptide functionalization did not obscure the reporter signal, enabling detection of nanostructures bound to cell-surface antigens after washing steps. A representative example of all NS spectra is reported in Figure S6. The morphology and microstructure of the samples confirming the success of the functionalization process were obtained by TEM analysis. The images were recorded with an accelerating voltage of 200 kV. A carbon-supported copper grid, 400 mesh size, was used for preparation of the sample. The results are reported in Figure S7.

In Vitro Targeting. MDA-MB-231 breast cancer cells, which express a high level of PD-L1, grew in T75 flasks until approximately 80% confluency was reached. Cells were then removed with trypsin, washed with PBS, and 10,000 cells were seeded in eight-chamber slides. After a 24 hour incubation period, the cells were incubated with varying concentrations of NS solutions for a period of 2 hours at 37°C. After the treatment, the cells were washed twice with phosphate buffer saline solution and immediately fixed with 2% paraformaldehyde for one hour, after which they were air-dried.

SERS measurements were conducted using a micro-Raman spectrometer with a 20 $\times$ objective and excitation at 633 nm using a He–Ne laser. A spectrum was collected for each cell using 10 seconds of laser irradiation (1 mW on the sample) in a static configuration. Spectra were recorded at approximately 1500 cm^{-1} , where the two main peaks of the SERS reporter TR-SH are located. A MATLAB (MATLAB R2022a, The MathWorks Inc., Natick, MA, USA) code was used to determine Pearson's correlation coefficient of each spectrum, after subtraction of the spectral background, compared to the spectrum of the SERS reporter TR-SH. A Pearson correlation coefficient larger than 0.8 for the spectrum recorded from a cell was considered indicative of the presence of the NS and, therefore, of successful targeting of the cell.

RESULTS AND DISCUSSION

Molecular Dynamics Calculations

Molecular Dynamics Simulation in the PD-1/PD-L1 Interaction. Extensive research has mapped the key interacting residues, often referred to as 'active spots', responsible for the stability and functional integrity of the PD-1/PD-L1 complex. Furthermore, the importance of a central region called 'hydrophobic core' (made up of Tyr56, Met115, Ile116, Ala121 and Tyr123) in mediating and strengthening this crucial interaction has been firmly established.⁵¹ Our project uses this knowledge to develop a peptide-based theranostic tool that competes effectively with PD-1 to bind to PD-L1, allowing rapid tumor recognition and restoration of T cell activity against cancer cells.

As described earlier, the starting point is the determination by MD simulation of the key amino acids of the active spots of PD-L1 that are those mainly involved in the binding of PD-1/PD-L1. MD simulation was performed to evaluate how the interaction found by the crystallographic structure evolves over time in solution, which is a more realistic starting point for our study. It should be taken into account that in this interaction, instead of considering native PD-1, we considered a variant PD-1 (m-PD-1) whose affinity for PD-L1 is 74000 times higher than that of native.^{73–75} As shown in Figure 1, the

m-PD-1 residues that most frequently contact the PD-L1 active spots are His64, Val66, His68, Asp77, Thr78, Gly124, Ile126, Ile132, Gln133, Ile134, Glu136, and Arg139. On this basis, we designed two peptides: PTP and sPTP (Table 1), whose 16-residue core reproduces this set of contact residues by residue type; the explicit residue-to-residue correspondence between the m-PD-1 contact residue and the peptide sequence is given in Scheme S1. Because targeting PD-L1 is achieved by a peptide monolayer and not by an isolated peptide, the design reproduces the chemical identity of the contact residues rather than their exact spatial arrangement, and all except Asp77 are represented in the core sequence. The remaining positions were selected to act as spacers and maximize the hydrophobic character of the peptides, thereby favoring the interaction with the hydrophobic active site of PD-L1; in particular, the Trp and Tyr residues also enable UV–vis quantification of the peptides, while the presence of a linker ($\text{NH}(\text{CH}_2\text{CH}_2\text{O})_2\text{CH}_2\text{CO-Cys}$ or Cys-Lys3-Gly2 , see Table 1) allows the chemisorption on the gold surface and therefore NS functionalization.

Molecular Dynamics Simulation of Peptides and PD-L1 in Solution. The two proposed peptides PTP and sPTP carry the PD-1 residues that are more responsible for the interaction with the active spots of PD-L1 (such as Leu, Glu, Ile, Gly, Val Arg, His). As shown in Table 1, these peptides have the same residues with different primary structures; this is to investigate the arrangement of the residues to optimize overlap with the active spots of PD-L1. Furthermore, to investigate the role of the primary structure in greater detail, a peptide with the same primary sequence of PTP but carrying the Cys residue to the N-terminal end (rPTP), which is able to link to the gold surface in reverse orientation with respect to PTP, has been studied.

In our systems, peptides are linked to a nanoparticle, and, most likely, PD-L1 interacts with an assembly of peptides arranged in a parallel way. To this end, as described above, it is advisable, in a preliminary step, regarding MD simulation in solution, to consider the interaction of PD-L1 with a peptide cluster rather than with a peptide alone.

One key point in the design of nanoparticles that carry peptides is the identification of the suitable linker to the surface. In previous papers, we attached the peptides to the nanoparticles by long poly(ethylene) chains and demonstrated the need to add charged moieties to increase the affinity of the peptides.^{43,62} In this case, to evaluate the effect of the primary structure only, we have decided to use short linkers: $-\text{NH}(\text{CH}_2\text{CH}_2\text{O})_2\text{CH}_2\text{CO-Cys-}$ for the C-terminal end and $-\text{Cys-Lys}_3\text{-Gly}_2\text{-}$ for the N-terminal end of the peptides (see Table 1).

First, we performed MD simulations of PD-L1 and a cluster of three parallel peptides to mimic the arrangement of the peptides on the surface. It represents an initial screening to evaluate the performance of the proposed peptides. The rationale for this choice is physically motivated by the fact that a single peptide in solution retains full conformational freedom and explores compact and folded structures that do not reflect the constrained, extended conformations adopted upon surface immobilization at high local density. As a result of the linkage to AuNp, peptides are forced to assume the same orientation, and the cluster configuration enforces interchain contacts and reduces the amount of accessible conformational space, producing starting structures that more faithfully represent the operational state of the peptide on the nanostructure surface. In

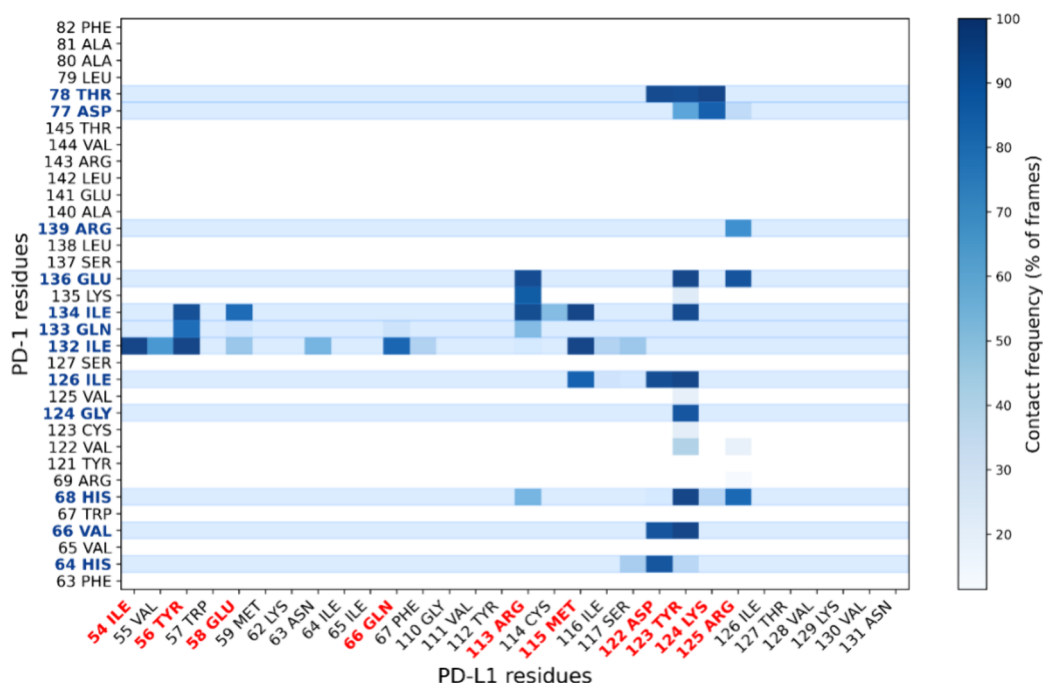


Figure 1. Bidimensional histogram of the residues in contact between the active region of PD-L1 and m-PD-1. The sidebar of the histogram provides a quantitative evaluation of the frequency of contacts in the last 20 ns of the MD simulation. In red are the active spots of PD-L1, while in blue, the m-PD-1 residues that are more in contact with PD-L1 are shown.

this way, it is possible to evaluate, in a first attempt, at relatively reduced time costs, the interaction of PD-L1 with a portion of a monolayer-like system.

During simulations, we examined the energy interaction of the peptide triplet with the PD-L1 active region. The data obtained are reported in Figure 2, while snapshots of each triplet that interact with the PD-L1 domain are in Figure 3. The reported energy values represent the averages over 20 independent replicas per system; the spread across replicas was consistent within each system, with standard deviations below 15% of the mean values, confirming the robustness of the relative ranking. As shown in Figure 2A, the interaction energy is approximately four times stronger for the PTP peptide cluster than for sPTP, thus indicating that the PTP triplet is more able to interact with the active spots of PD-L1. At the same time, the interaction energy of the rPTP assembly with PD-L1 is very small, suggesting that the peptide orientation (in the case of the tool that deals with peptides linked to a surface) plays an important role in the efficacy of NS. It is interesting to note that, by comparing the data in Figure 2A,B, the main contribution to the interaction energy is due to the van der Waals one, suggesting that the peptide interaction probably occurs primarily with the PD-L1 residues of the so-called 'hydrophobic core' (see above)⁵¹ in the active region.

Indeed, in Figure 3, it is interesting to note that the secondary structures and the aggregation among peptides in the three triplets are different, as well as the interaction with the active spots of PD-L1. This finding indicates the strong interconnection among the peptide sequence, the aggregation of the peptide triplet and its interaction with PD-L1.

To our knowledge, this is the first time this type of analysis has been performed because normally^{34,46,53,76,77} the interaction of PD-L1 with a single peptide is performed. In our case, the

results obtained for the interaction between a single peptide and PD-L1 in solution, reported in the Supporting Information (Figure S8), show a reversed ranking with respect to what is observed for the cluster: sPTP appears to interact more favorably with the PD-L1 region containing the active spots, while PTP shows a weaker interaction. This counterintuitive result highlights how the conformational ensemble sampled by an isolated peptide in solution poorly represents the functional state of the peptide on the nanostructure surface and reinforces the need to adopt a cluster-based model for a more physically meaningful prediction of target performance when the peptides are chemisorbed to AuNPs. This approach can be used as a first rapid screening tool for the evaluation of the proposed peptide effectiveness, avoiding the need for more time-consuming MD simulations of the peptide monolayer on the surface.

Finally, it should be noted that these interaction energies represent only an estimated binding free energies; these values would require methods such as MM-PBSA or umbrella sampling, which, however, entail substantial computational cost and demand significant resources in terms of simulation time and hardware, limiting their applicability as routine screening tools in early-stage design workflows. The framework proposed here is intentionally conceived as a computationally accessible yet physically meaningful approach, capable of ranking peptide candidates and identifying dominant interaction modes prior to experimental validation, without the overhead of exhaustive free-energy calculations.

Molecular Dynamics Simulation of PD-L1 and Peptides on the Surface. Simulations were performed on a peptide monolayer on a gold surface. Because MD simulations in solution do not adequately describe the systems under investigation (they do not take into account interactions between

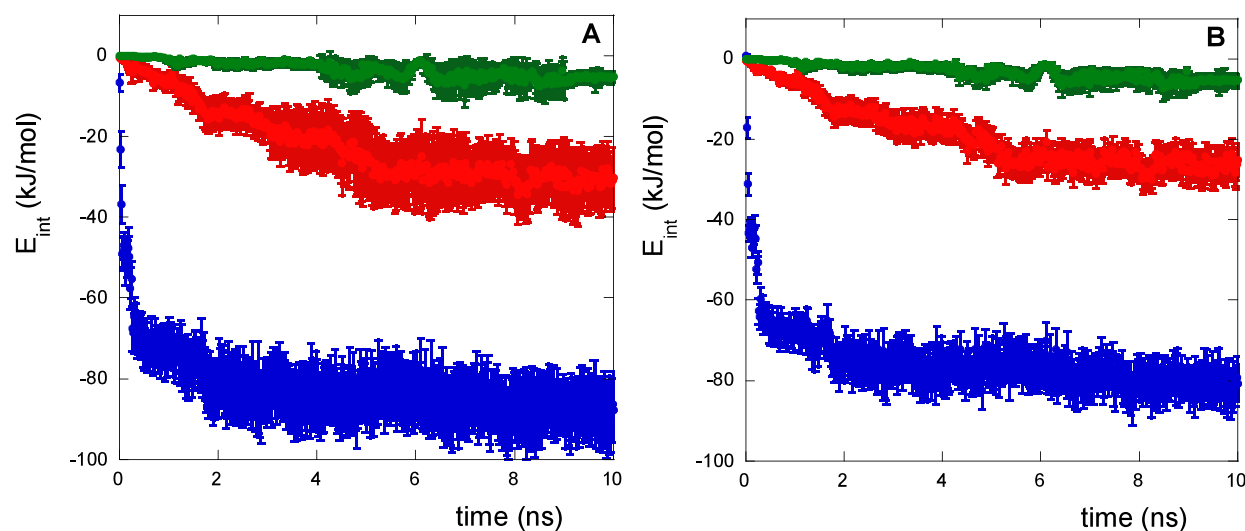


Figure 2. Interaction energies (E_{int}) of PD-L1 with the trimer PTP (blue), sPTP (red), and rPTP (green). (A) Electrostatic plus the vdW contribution. (B) van der Waals contribution only.

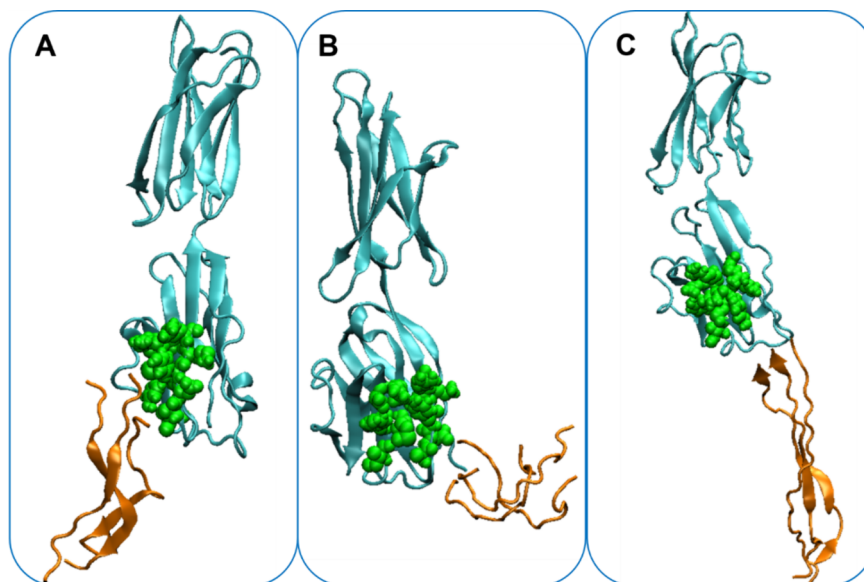


Figure 3. Image of binding near the active spots of the PD-L1 (green) and (A) PTP, (B) sPTP, and (C) rPTP trimers (orange). In the peptide cluster, C is cyan, O is red, N is blue, and S is yellow. PD-L1 is cyan. The images were obtained using VMD software.

larger numbers of peptides), the first part of the project focused on analyzing the peptide monolayers to identify any differences in their structural arrangement.

Two 100 ns replicas were performed for each simulation. As an example, snapshots of the last frame of one replica of each simulation are presented in Figure 4.

As can be observed in Figure 4, all peptides display a proclivity to aggregation on the gold surface, but with different features. While, for example, PTP@NS shows a compact but not flat peptide monolayer, sPTP@NS shows a very compact and uniform peptide layer. At the same time, rPTP@NS shows an inhomogeneous peptide monolayer. These could be explained by considering that the three peptides assume different

secondary structures and aggregates, similarly to what is observed in solution (see above).

This different behavior has been confirmed by calculating the solvent-accessible surface area (SASA) per peptide (Table 3) and the number of contacts among the peptides. Results are shown in Figure 5.

As illustrated in Figure 5A, the solvent-accessible surface area (SASA) per peptide shows disparate patterns for the peptides. Notably, a more pronounced reduction in SASA is evident for the peptide monolayers in sPTP@NS and PTP@NS compared to the rPTP@NS one. At the same time, the number of contacts per peptide, shown in Figure 5B, indicates that the peptides in sPTP@NS interact more strongly with each other than in

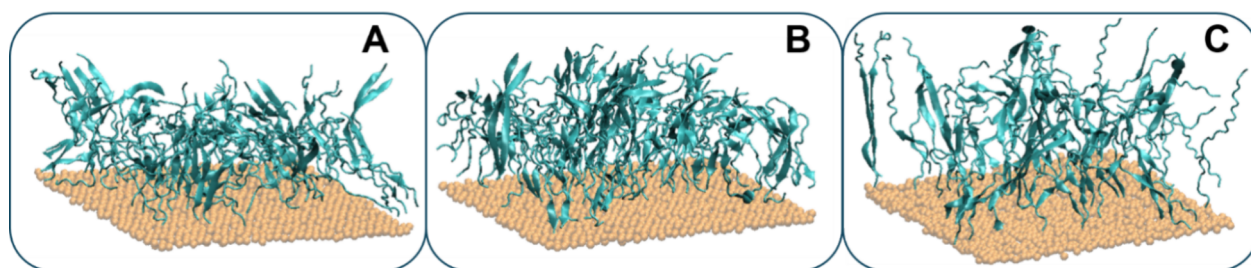


Figure 4. Images of the last frame of simulation of (A) PTP@NS; (B) sPTP@NS; and (C) rPTP@NS.

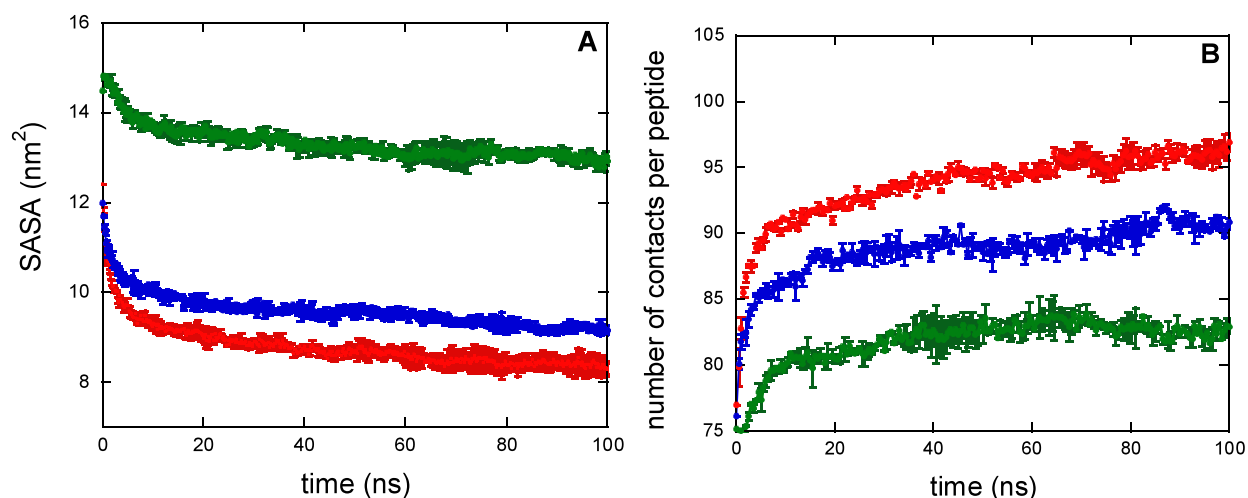


Figure 5. (A) SASA and (B) number of contacts per peptide of PTP@NS (blue), sPTP@NS (red), and rPTP@NS (green). The data reported represent the average of two simulations.

PTP@NS and rPTP@NS. These data indicate that sPTP peptides form a homogeneous compact structure on the surface having a greater propensity to aggregate on the surface. It is interesting to note that in the case of rPTP@NS, SASA is always the highest, and the number of contacts among peptides is very low compared to that of sPTP@NS, indicating that this peptide has a lower tendency to aggregate and there are void spaces on the surface. This last result is in contrast with that obtained with PTP@NS, in which the difference is in the orientation of the peptide only. In the case of PTP@NS, we obtain a much more homogeneous and compact monolayer, even if it is less than that of sPTP@NS.

This result points out the key role not only of the peptide primary sequence but also, importantly, of peptide orientation with respect to the surface in ruling the overall surface morphology and characteristics.

Subsequently, MD simulations were performed to evaluate the interaction between the peptides on NS and PD-L1. To this end, the simulated surface was ideally divided into four quadrants (Figure S1), and the protein was initially placed in the center of each quadrant. This approach was selected to avoid any potential bias associated with the initial positioning of PD-L1 in the simulations.

As shown in Figure 6, in the case of sPTP@NS, there is a minimal variation in the initial and final positions of PD-L1, with this protein remaining within the same quadrant

throughout the simulation and no preferential region was identified; this result may be indicative of a certain degree of non-specificity in the interaction. On the contrary, in the case of PTP@NS, the monolayer exhibited a different behavior. In three of the four quadrants, PD-L1 was observed to migrate to the same portion of the monolayer over the course of the simulations, regardless of the initial starting point. This strongly suggests the presence of a specific binding site and a significant extent of interaction specificity. Interestingly, in the case of rPTP@NS, regardless of the starting point, not only there is no shift from the initial and final positions of PD-L1 (as for sPTP@NS), but also, it is not able to insert into the monolayer and instead interact with the monolayer with the lower part of the binding region, similar to what is found in solution (see Figure 3C). The very significant reduction in PD-L1 interaction for rPTP@NS can be rationalized at the structural level by considering the accessibility of the key binding residues. In PTP, the C-terminal O2oc-Cys linker anchors the peptide to the gold surface with functional residues, particularly those that mimic hydrophobic core contacts (Leu, Tyr, Ile, Val), projected toward the solvent and available for PD-L1 engagement. In rPTP, the N-terminal Cys attachment reverses this geometry: the same residues are buried near the gold surface, while the C-terminal end is solvent-exposed, but does not carry the functionally relevant contacts. This orientation-dependent shielding of the binding motif, captured quantitatively by the near-zero

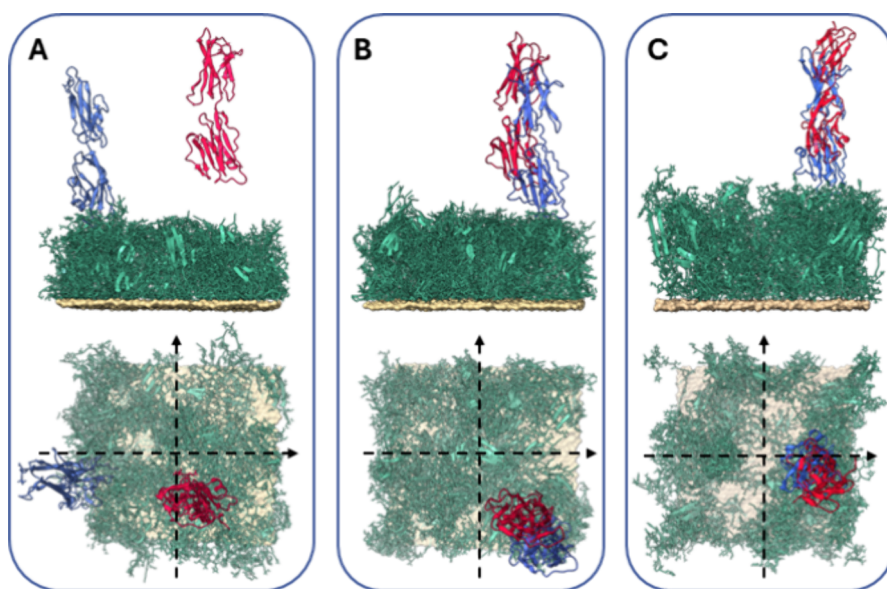


Figure 6. Side and top views of the first (red) and last (blue) frames of PD-L1 during MD simulation with (A) PTP@NS; (B) sPTP@NS; and (C) rPTP@NS monolayers.

interaction energy and contact map in Figures 7 and 8C, underscores that surface-anchoring geometry is as critical a design parameter as the amino acid sequence itself.

The effectiveness of the interaction has been evaluated by calculating the interaction energy in terms of electrostatic and van der Waals contributions (see above) between the active spots of PD-L1 and each monolayer. As shown in Figure 7A, the results closely resemble those obtained in solution with peptide assemblies. Also, in this case, this interaction is strongest in the case of PTP@NS, more than one-half less for sPTP@NS, while it is almost zero for rPTP@NS. Furthermore, as in this case, the greater contribution of the interaction energy is due to van der Waals (Figure 7B).

Evaluation of the number of contacts between peptides in the NS and the active spots of PD-L1 confirms these findings.

As shown in Figure 8, in fact, the number of contacts between the active spots of PD-L1 and PTP@NS is higher than the other two samples, demonstrating a more extensive interaction, which could lead to a better affinity for the target. Furthermore, it can be observed that, in the case of PTP@NS, interactions occur with specific residues known to contribute to the formation of the hydrophobic core at the active spots of PD-L1 (Tyr56, Met115, Ile116, Ala121, and Tyr123).⁵¹ This finding further validates the successful engineering of the peptide, indicating that the most significant interactions, those that confer selectivity toward PD-L1, are indeed these specific interactions.

This result highlights the importance of the sequence and orientation in pointing out the targeting peptide linked to a surface for protein recognition.

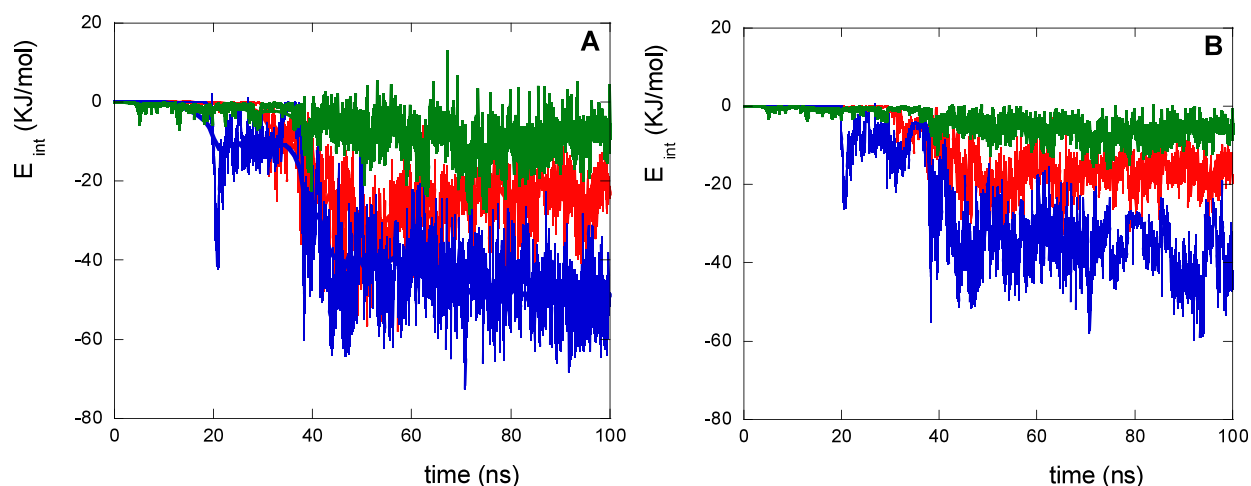


Figure 7. Interaction energies (E_{int}) of PD-L1 with PTP@NS (blue), rPTP@NS (green), and sPTP@NS (red). (A) Electrostatic plus van der Waals contribution. (B) van der Waals contribution only.

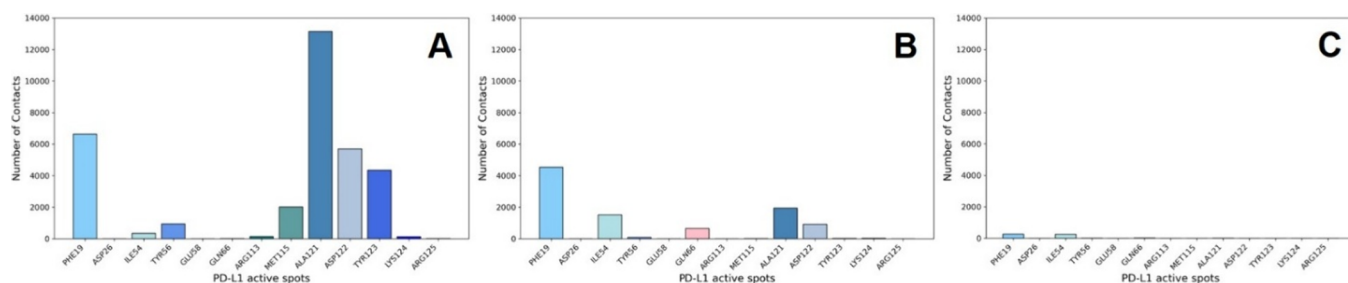


Figure 8. Number of contacts of PD-L1 active spots with the peptide monolayer in (A) PTP@NS; (B) sPTP@NS, and (C) rPTP@NS in the last 20 ns of MD simulations.

Subsequently, these results will be compared with the corresponding experimental data.

In Vitro Assay. Following the procedure reported in the **Materials and Methods** section, the NSs were prepared and their targeting activity was tested against MDA-MB-231 cells over-expressing PD-L1 by means of SERS measurements. As shown in **Figure 9**, the highest affinity for PD-L1 was observed at an NS concentration of 100 pM for PTP@NS (approximately 85%), while the lowest (23%) was observed for rPTP@NS.

The results of these experiments corroborate the hypothesis postulated by the MD simulations and confirm the pivotal role of the interactions between the PD-L1 active spots and the monolayer, thus validating the MD simulation approach employed. It should be noted that the SERS-based targeting assay provides evaluation of cellular targeting efficiency, that is, a quantitative assessment of nanostructure association with PD-L1-expressing cells and should not be interpreted as a direct measurement, to obtain binding affinity parameters such as K_d or IC_{50} . In this context, PTP@NS, sPTP@NS, and rPTP@NS act as an internal control: the three systems share the same gold nanostructure platform and the same SERS reporter and differ only in peptide sequence and/or anchoring orientation, so the markedly different targeting efficiencies observed support the conclusion that peptide organization and orientation critically affect PD-L1 recognition. In other words, the use of the scrambled sequence sPTP and the reverse-orientation peptide rPTP as internal controls within the same experimental set provides

a first level of selectivity evaluation. Indeed, this study illustrates the need to consider both the structural characteristics of the monolayer and its interactions with the target when developing high-affinity peptides.

CONCLUSIONS

Immune checkpoint inhibition, particularly targeting PD-L1, is a key strategy in cancer immunotherapy. In this study, we developed and characterized gold nanostructures functionalized with designed peptides targeting PD-L1, using a combined approach of molecular dynamics simulations and experimental validation. The amino acid composition of the peptides has been chosen by individuating the PD-1 residues that interact with the active spots of PD-L1. Sequences with identical composition but different orientations with respect to the gold surface along with a scrambled sequence were used to isolate the effects of residue arrangement and surface feature, thus allowing direct testing of how sequence organization on the nanostructure influences functional recognition. The simulations are designed to answer a specific question: peptides, under conditions that reflect surface immobilization and high local density, maintain accessibility and persistent contacts with active spots of PD-L1. By explicitly analyzing the PD-L1/peptides contact regions, binding persistence, and shielding effects under crowding conditions, the computational framework directly probes the determinants of effective surface-mediated targeting.

Furthermore, our study underscores the importance of considering both the peptide sequence design and monolayer organization in the rational development of a PD-L1-targeting peptide trimer. It integrates surface-based molecular modeling to capture the complexity of multivalent and collective interactions.

In conclusion, this work provides an experimentally validated approach for the rational design of peptide-functionalized nanostructures with enhanced immune checkpoint targeting capabilities. By bridging computational modeling with experimental validation, our approach paves the way for the development of next-generation peptide-based nanotheranostic platforms, offering promising avenues for improved cancer treatment strategies. It should be pointed out that this work provides a valid protocol to perform rational advances in this field. However, in the examples considered, further experiments (proteolytic digestions, peptide stability tests, affinity parameter measurements) should be taken into account for a fully final experimental validation of the engineered nanoparticle proposed, which is, however, out of the scope of this work.

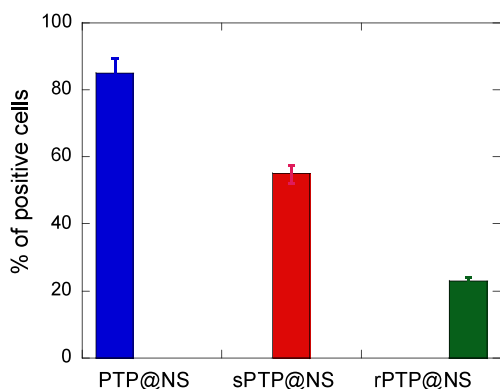


Figure 9. Activity of PTP@NS (blue), sPTP@NS (red), and rPTP@NS (green) for the targeting of MDA-MB-231 over-expressing PD-L1. The percentage of the cells with a positive target, namely, with Pearson's correlation coefficient above the 0.8 threshold, is plotted at a 100 pM concentration of NS incubation.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsabm.6c01076>.

Scheme reproducing the amino acid sequences of mPD-1 and the derived peptides PTP and sPTP, representative scheme of the quadrant division of the surface for the MD simulations in dials, analytical HPLC chromatogram and the corresponding ESI-TOF mass spectrum of all of the peptides, SERS spectrum of PTP@NS, TEM images of all of the functionalized NS and of gold NPs, images of the beginning of the MD simulation of the PTP triplet in solution in the presence of PD-L1, and two-dimensional contact maps between a single PTP (left) or sPTP (right) peptide and the active spots of PD-L1 (DOCX)

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curation, writing—review and editing, and visualization. L.L.: Methodology, investigation, and data curation. I.C.: Methodology, formal analysis, and investigation. F.R.: Conceptualization, resources, and writing—review and editing. M.M.: Funding, conceptualization, methodology, resources, writing—review and editing, and supervision. C.M.: Validation, formal analysis, data curation, writing—original draft preparation, and writing—review and editing. A.P.: Conceptualization, methodology, software, resources, writing—original draft preparation, writing—review and editing, and visualization. All authors have read and agreed to the published version of the manuscript.

Notes

The authors declare no competing financial interest.

■ REFERENCES

- (1) Silva, G. A. Introduction to Nanotechnology and Its Applications to Medicine. *Surg. Neurol.* **2004**, 61 (3), 216–220.
- (2) Haleem, A.; Javaid, M.; Singh, R. P.; Rab, S.; Suman, R. Applications of Nanotechnology in Medical Field: A Brief Review. *Glob. Health J.* **2023**, 7 (2), 70–77.
- (3) Dykman, L.; Khlebtsov, N. Gold Nanoparticles in Biomedical Applications: Recent Advances and Perspectives. *Chem. Soc. Rev.* **2012**, 41 (6), 2256–2282.
- (4) Boisselier, E.; Astruc, D. Gold Nanoparticles in Nanomedicine: Preparations, Imaging, Diagnostics, Therapies and Toxicity. *Chem. Soc. Rev.* **2009**, 38 (6), 1759.
- (5) Giljohann, D. A.; Seferos, D. S.; Daniel, W. L.; Massich, M. D.; Patel, P. C.; Mirkin, C. A. Gold Nanoparticles for Biology and Medicine. *Angew. Chem. Int. Ed.* **2010**, 49 (19), 3280–3294.
- (6) Slavin, Y. N.; Asnis, J.; Häfeli, U. O.; Bach, H. Metal Nanoparticles: Understanding the Mechanisms behind Antibacterial Activity. *J. Nanobiotechnol.* **2017**, 15 (1), 65.
- (7) Cagno, V.; Andreozzi, P.; D'Alicarnasso, M.; Jacob Silva, P.; Mueller, M.; Galloux, M.; Le Goffic, R.; Jones, S. T.; Vallino, M.; Hodek, J.; Weber, J.; Sen, S.; Janeček, E.-R.; Bekdemir, A.; Sanavio, B.; Martinelli, C.; Donalisio, M.; Rameix Welti, M.-A.; Eleouet, J.-F.; Han, Y.; Kaiser, L.; Vukovic, L.; Tapparel, C.; Král, P.; Krol, S.; Lembo, D.; Stellacci, F. Broad-Spectrum Non-Toxic Antiviral Nanoparticles with a Virucidal Inhibition Mechanism. *Nat. Mater.* **2018**, 17 (2), 195–203.
- (8) Conde, J.; Dias, J. T.; Grazú, V.; Moros, M.; Baptista, P. V.; de la Fuente, J. M. Revisiting 30 Years of Biofunctionalization and Surface Chemistry of Inorganic Nanoparticles for Nanomedicine. *Front. Chem.* **2014**, 2, 48.
- (9) Jokerst, J. V.; Lobovkina, T.; Zare, R. N.; Gambhir, S. S. Nanoparticle PEGylation for Imaging and Therapy. *Nanomedicine* **2011**, 6 (4), 715–728.
- (10) Mayer, K. M.; Hafner, J. H. Localized Surface Plasmon Resonance Sensors. *Chem. Rev.* **2011**, 111 (6), 3828–3857.
- (11) Schlücker, S. Surface-Enhanced Raman Spectroscopy: Concepts and Chemical Applications. *Angew. Chem. Int. Ed.* **2014**, 53 (19), 4756–4795.
- (12) Mosier-Boss, P. Review of SERS Substrates for Chemical Sensing. *Nanomaterials* **2017**, 7 (6), 142.
- (13) Huang, X.; El-Sayed, M. A. Gold Nanoparticles: Optical Properties and Implementations in Cancer Diagnosis and Photothermal Therapy. *J. Adv. Res.* **2010**, 1 (1), 13–28.
- (14) Jaque, D.; Martínez Maestro, L.; del Rosal, B.; Haro-Gonzalez, P.; Benayas, A.; Plaza, J. L.; Martín Rodríguez, E.; García Solé, J. Nanoparticles for Photothermal Therapies. *Nanoscale* **2014**, 6 (16), 9494–9530.
- (15) Kelkar, S. S.; Reineke, T. M. Theranostics: Combining Imaging and Therapy. *Bioconjug. Chem.* **2011**, 22 (10), 1879–1903.

- (16) Mura, S.; Couvreur, P. Nanotheranostics for Personalized Medicine. *Adv. Drug Deliv. Rev.* **2012**, *64* (13), 1394–1416.
- (17) Hayashi, H.; Nakagawa, K. Combination Therapy with PD-1 or PD-L1 Inhibitors for Cancer. *Int. J. Clin. Oncol.* **2020**, *25* (5), 818–830.
- (18) Jiang, Y.; Chen, M.; Nie, H.; Yuan, Y. PD-1 and PD-L1 in Cancer Immunotherapy: Clinical Implications and Future Considerations. *Hum. Vaccin. Immunother.* **2019**, *15* (5), 1111–1122.
- (19) Blank, C.; Mackensen, A. Contribution of the PD-L1/PD-1 Pathway to T-Cell Exhaustion: An Update on Implications for Chronic Infections and Tumor Evasion. *Cancer Immunol. Immunother.* **2007**, *56* (5), 739–745.
- (20) Zou, M.; Liu, W.; Li, C.; Zheng, D.; Zeng, J.; Gao, F.; Ye, J.; Zhang, X. A Multifunctional Biomimetic Nanoplatform for Relieving Hypoxia to Enhance Chemotherapy and Inhibit the PD-1/PD-L1 Axis. *Small* **2018**, *14* (28), 1801120.
- (21) Jin, X.; Zhang, S.; Liang, J.; Zhang, S.; Qin, Y.; Huang, Q.; Liu, C.; Zhang, X. A PD-L1-targeting Regulator for Metabolic Reprogramming to Enhance Glutamine Inhibition-Mediated Synergistic Antitumor Metabolic and Immune Therapy. *Adv. Mater.* **2024**, *36* (6), 2309094.
- (22) Riella, L. V.; Paterson, A. M.; Sharpe, A. H.; Chandraker, A. Role of the PD-1 Pathway in the Immune Response. *Am. J. Transplant.* **2012**, *12* (10), 2575–2587.
- (23) Lu, D.; Ni, Z.; Liu, X.; Feng, S.; Dong, X.; Shi, X.; Zhai, J.; Mai, S.; Jiang, J.; Wang, Z.; Wu, H.; Cai, K. Beyond T Cells: Understanding the Role of PD-1/PD-L1 in Tumor-Associated Macrophages. *J. Immunol. Res.* **2019**, *2019*, 1919082.
- (24) Liu, W.; Jin, H.; Chen, T.; Zhang, G.; Lai, S.; Liu, G. Investigating the Role of the N-Terminal Loop of PD-1 in Binding Process Between PD-1 and Nivolumab via Molecular Dynamics Simulation. *Front. Mol. Biosci.* **2020**, *7*, 574759.
- (25) Liu, K.; Tan, S.; Chai, Y.; Chen, D.; Song, H.; Zhang, C. W.-H.; Shi, Y.; Liu, J.; Tan, W.; Lyu, J.; Gao, S.; Yan, J.; Qi, J.; Gao, G. F. Structural Basis of Anti-PD-L1 Monoclonal Antibody Avelumab for Tumor Therapy. *Cell Res.* **2017**, *27* (1), 151–153.
- (26) Zak, K. M.; Grudnik, P.; Magiera, K.; Dömling, A.; Dubin, G.; Holak, T. A. Structural Biology of the Immune Checkpoint Receptor PD-1 and Its Ligands PD-L1/PD-L2. *Structure* **2017**, *25* (8), 1163–1174.
- (27) Chen, Y.; Liu, P.; Gao, F.; Cheng, H.; Qi, J.; Gao, G. F. A Dimeric Structure of PD-L1: Functional Units or Evolutionary Relics? *Protein Cell* **2010**, *1* (2), 153–160.
- (28) Skalniak, L.; Zak, K. M.; Guzik, K.; Magiera, K.; Musielak, B.; Pachota, M.; Szelazek, B.; Kocik, J.; Grudnik, P.; Tomala, M.; Krzanik, S.; Pyrc, K.; Dömling, A.; Dubin, G.; Holak, T. A. Small-Molecule Inhibitors of PD-1/PD-L1 Immune Checkpoint Alleviate the PD-L1-Induced Exhaustion of T-Cells. *Oncotarget* **2017**, *8* (42), 72167–72181.
- (29) Cheng, X.; Veverka, V.; Radhakrishnan, A.; Waters, L. C.; Muskett, F. W.; Morgan, S. H.; Huo, J.; Yu, C.; Evans, E. J.; Leslie, A. J.; Griffiths, M.; Stubberfield, C.; Griffin, R.; Henry, A. J.; Jansson, A.; Ladbury, J. E.; Ikemizu, S.; Carr, M. D.; Davis, S. J. Structure and Interactions of the Human Programmed Cell Death 1 Receptor. *J. Biol. Chem.* **2013**, *288* (17), 11771–11785.
- (30) Boutros, C.; Tarhini, A.; Routier, E.; Lambotte, O.; Ladurie, F. L.; Carbonnel, F.; Izzeddine, H.; Marabelle, A.; Champiat, S.; Berdelou, A.; Lanoy, E.; Texier, M.; Libenciuc, C.; Eggermont, A. M. M.; Soria, J.-C.; Mateus, C.; Robert, C. Safety Profiles of Anti-CTLA-4 and Anti-PD-1 Antibodies Alone and in Combination. *Nat. Rev. Clin. Oncol.* **2016**, *13* (8), 473–486.
- (31) Wang, H.; Yao, H.; Li, C.; Shi, H.; Lan, J.; Li, Z.; Zhang, Y.; Liang, L.; Fang, J.-Y.; Xu, J. HIP1R Targets PD-L1 to Lysosomal Degradation to Alter T Cell-Mediated Cytotoxicity. *Nat. Chem. Biol.* **2019**, *15* (1), 42–50.
- (32) Kamalinia, G.; Engel, B. J.; Srinivasamani, A.; Grindel, B. J.; Ong, J. N.; Curran, M. A.; Takahashi, T. T.; Millward, S. W.; Roberts, R. W.; Roberts, R. W. MRNA Display Discovery of a Novel Programmed Death Ligand 1 (PD-L1) Binding Peptide (a Peptide Ligand for PD-L1). *ACS Chem. Biol.* **2020**, *15* (6), 1630–1641.
- (33) Magiera-Mularz, K.; Skalniak, L.; Zak, K. M.; Musielak, B.; Rudzinska-Szostak, E.; Berlicki, L.; Kocik, J.; Grudnik, P.; Sala, D.; Zarganes-Tzitzikas, T.; Shaabani, S.; Dömling, A.; Dubin, G.; Holak, T. A. Bioactive Macrocyclic Inhibitors of the PD-1/PD-L1 Immune Checkpoint. *Angew. Chem. Int. Ed.* **2017**, *56* (44), 13732–13736.
- (34) Rui, M.; Zhang, W.; Mi, K.; Ni, H.; Ji, W.; Yu, X.; Qin, J.; Feng, C. Design and Evaluation of α -Helix-Based Peptide Inhibitors for Blocking PD-1/PD-L1 Interaction. *Int. J. Biol. Macromol.* **2023**, *253*, 126811.
- (35) Liang, J.; Luo, G.; Chen, W.; Zhang, X. Recent Advances in Engineered Materials for Immunotherapy-Involved Combination Cancer Therapy. *Adv. Mater.* **2021**, *33* (31), 2007630.
- (36) Ayass, M. A.; Tripathi, T.; Griko, N.; Okay, T.; Ramankutty Nair, R.; Zhang, J.; Zhu, K.; Melendez, K.; Pashkov, V.; Abi-Mosleh, L. Dual Checkpoint Aptamer Immunotherapy: Unveiling Tailored Cancer Treatment Targeting CTLA-4 and NKG2A. *Cancers (Basel)* **2024**, *16* (5), 1041.
- (37) Bertrand, P. Aptamers Targeting the PD-1/PD-L1 Axis: A Perspective. *J. Med. Chem.* **2023**, *66* (16), 10878–10888.
- (38) Wu, Q.; Jiang, L.; Li, S.; He, Q.; Yang, B.; Cao, J. Small Molecule Inhibitors Targeting the PD-1/PD-L1 Signaling Pathway. *Acta Pharmacol. Sin.* **2021**, *42* (1), 1–9.
- (39) Zhan, M.-M.; Hu, X.-Q.; Liu, X.-X.; Ruan, B.-F.; Xu, J.; Liao, C. From Monoclonal Antibodies to Small Molecules: The Development of Inhibitors Targeting the PD-1/PD-L1 Pathway. *Drug Discov. Today* **2016**, *21* (6), 1027–1036.
- (40) Jiang, Q.; Yao, F.; An, Y.; Lai, X.; Li, X.; Yu, Z.; Yang, X.-D. Novel Nanotherapeutics for Cancer Immunotherapy by Albumin Nanoparticles Functionalized with PD-1 and PD-L1 Aptamers. *Cancer Nanotechnol.* **2024**, *15* (1), 3.
- (41) Linde, C.; Chien, Y.-T.; Chen, Z.; Mu, Q. Nanoparticle-Enhanced PD-1/PD-L1 Targeted Combination Therapy for Triple Negative Breast Cancer. *Front. Oncol.* **2024**, *14*, 1393492.
- (42) Song, S.; Shim, M. K.; Yang, S.; Lee, J.; Yun, W. S.; Cho, H.; Moon, Y.; Min, J. Y.; Han, E. H.; Yoon, H. Y.; Kim, K. All-in-One Glycol Chitosan Nanoparticles for Co-Delivery of Doxorubicin and Anti-PD-L1 Peptide in Cancer Immunotherapy. *Bioact. Mater.* **2023**, *28*, 358–375.
- (43) Giannetti, M.; Gobbo, M.; Litti, L.; Caligiuri, I.; Rizzolio, F.; Meneghetti, M.; Mazzuca, C.; Palleschi, A. Unraveling the Specific Recognition Between PD-L1 and Engineered CLP002 Functionalized Gold Nanostructures: MD Simulation Studies. *Molecules* **2025**, *30* (9), 2045.
- (44) Chang, H.; Liu, B.; Qi, Y.; Zhou, Y.; Chen, Y.; Pan, K.; Li, W.; Zhou, X.; Ma, W.; Fu, C.; Qi, Y.; Liu, L.; Gao, Y. Blocking of the PD-1/PD-L1 Interaction by a D-Peptide Antagonist for Cancer Immunotherapy. *Angew. Chem. Int. Ed.* **2015**, *54* (40), 11760–11764.
- (45) Sasikumar, P. G.; Ramachandra, R. K.; Adurthi, S.; Dhudashiya, A. A.; Vadlamani, S.; Vemula, K.; Vunnum, S.; Satyam, L. K.; Samiulla, D. S.; Subbarao, K.; Nair, R.; Shrimali, R.; Gowda, N.; Ramachandra, M. A Rationally Designed Peptide Antagonist of the PD-1 Signaling Pathway as an Immunomodulatory Agent for Cancer Therapy. *Mol. Cancer Ther.* **2019**, *18* (6), 1081–1091.
- (46) Moon, Y.; Shim, M. K.; Choi, J.; Yang, S.; Kim, J.; Yun, W. S.; Cho, H.; Park, J. Y.; Kim, Y.; Seong, J.-K.; Kim, K. Anti-PD-L1 Peptide-Conjugated Prodrug Nanoparticles for Targeted Cancer Immunotherapy Combining PD-L1 Blockade with Immunogenic Cell Death. *Theranostics* **2022**, *12* (5), 1999–2014.
- (47) Sakhawat, A.; Khan, M. U.; Khan, S.; Ahmed, N.; Alhegaili, A. S.; Alotaibi, B. S. PD-L1 Targeting in Triple Negative Breast Cancer: In Silico and in Vitro Validation of Wasp Venom Peptide MP-1. *Med. Oncol.* **2025**, *43* (1), 14.
- (48) Guzik, K.; Tomala, M.; Muszak, D.; Konieczny, M.; Hec, A.; Blaszkiewicz, U.; Pustula, M.; Butera, R.; Dömling, A.; Holak, T. A. Development of the Inhibitors That Target the PD-1/PD-L1 Interaction—A Brief Look at Progress on Small Molecules, Peptides and Macrocycles. *Molecules* **2019**, *24* (11), 2071.
- (49) Keefe, A. D.; Pai, S.; Ellington, A. Aptamers as Therapeutics. *Nat. Rev. Drug Discov.* **2010**, *9* (7), 537–550.

- (50) Liu, X.; Wang, T.; Wu, Y.; Tan, Y.; Jiang, T.; Li, K.; Lou, B.; Chen, L.; Liu, Y.; Liu, Z. Aptamer Based Probes for Living Cell Intracellular Molecules Detection. *Biosens. Bioelectron.* **2022**, *208*, 114231.
- (51) Zak, K. M.; Grudnik, P.; Guzik, K.; Zieba, B. J.; Musielak, B.; Dömling, A.; Dubin, G.; Holak, T. A. Structural Basis for Small Molecule Targeting of the Programmed Death Ligand 1 (PD-L1). *Oncotarget* **2016**, *7* (21), 30323–30335.
- (52) Vlieghe, P.; Lisowski, V.; Martinez, J.; Khrestchatsky, M. Synthetic Therapeutic Peptides: Science and Market. *Drug Discov. Today* **2010**, *15* (1–2), 40–56.
- (53) Miao, Q.; Zhang, W.; Zhang, K.; Li, H.; Zhu, J.; Jiang, S. Rational Design of a Potent Macrocyclic Peptide Inhibitor Targeting the PD-1/PD-L1 Protein–Protein Interaction. *RSC Adv.* **2021**, *11* (38), 23270–23279.
- (54) Fosgerau, K.; Hoffmann, T. Peptide Therapeutics: Current Status and Future Directions. *Drug Discov. Today* **2015**, *20* (1), 122–128.
- (55) Lau, J. L.; Dunn, M. K. Therapeutic Peptides: Historical Perspectives, Current Development Trends, and Future Directions. *Bioorg. Med. Chem.* **2018**, *26* (10), 2700–2707.
- (56) Gebauer, M.; Skerra, A. Engineered Protein Scaffolds as Next-Generation Antibody Therapeutics. *Curr. Opin. Chem. Biol.* **2009**, *13* (3), 245–255.
- (57) Craik, D. J.; Fairlie, D. P.; Liras, S.; Price, D. The Future of Peptide-based Drugs. *Chem. Biol. Drug Des.* **2013**, *81* (1), 136–147.
- (58) Rai, P.; Mallidi, S.; Zheng, X.; Rahmzadeh, R.; Mir, Y.; Elrington, S.; Khurshid, A.; Hasan, T. Development and Applications of Photo-Triggered Theranostic Agents. *Adv. Drug Deliv. Rev.* **2010**, *62* (11), 1094–1124.
- (59) Biscaglia, F.; Caligiuri, I.; Rizzolio, F.; Ripani, G.; Palleschi, A.; Meneghetti, M.; Gobbo, M. Protection against Proteolysis of a Targeting Peptide on Gold Nanostructures. *Nanoscale* **2021**, *13* (23), 10544–10554.
- (60) Gobbo, M.; Caligiuri, I.; Giannetti, M.; Litti, L.; Mazzuca, C.; Rizzolio, F.; Palleschi, A.; Meneghetti, M. SERS Nanostructures with Engineered Active Peptides against an Immune Checkpoint Protein. *Nanoscale* **2024**, *16* (10), 5206–5214.
- (61) Biscaglia, F.; Ripani, G.; Rajendran, S.; Benna, C.; Mocellin, S.; Bocchinfuso, G.; Meneghetti, M.; Palleschi, A.; Gobbo, M. Gold Nanoparticle Aggregates Functionalized with Cyclic RGD Peptides for Targeting and Imaging of Colorectal Cancer Cells. *ACS Appl. Nano Mater.* **2019**, *2* (10), 6436–6444.
- (62) Biscaglia, F.; Rajendran, S.; Conflitti, P.; Benna, C.; Soggiu, R.; Litti, L.; Mocellin, S.; Bocchinfuso, G.; Rosato, A.; Palleschi, A.; Nitti, D.; Gobbo, M.; Meneghetti, M. Enhanced EGFR Targeting Activity of Plasmonic Nanostructures with Engineered GE11 Peptide. *Adv. Healthc. Mater.* **2017**, *6* (23), 1700596.
- (63) Liu, H.; Zhao, Z.; Zhang, L.; Li, Y.; Jain, A.; Barve, A.; Jin, W.; Liu, Y.; Fetse, J.; Cheng, K. Discovery of Low-Molecular Weight Anti-PD-L1 Peptides for Cancer Immunotherapy. *J. Immunother. Cancer* **2019**, *7* (1), 270.
- (64) Mazzuca, C.; Di Napoli, B.; Biscaglia, F.; Ripani, G.; Rajendran, S.; Braga, A.; Benna, C.; Mocellin, S.; Gobbo, M.; Meneghetti, M.; Palleschi, A. Understanding the Good and Poor Cell Targeting Activity of Gold Nanostructures Functionalized with Molecular Units for the Epidermal Growth Factor Receptor. *Nanoscale Adv.* **2019**, *1* (5), 1970–1979.
- (65) Abraham, M.; Alekseenko, A.; Basov, V.; Bergh, C.; Briand, E.; Brown, A.; Doijade, M.; Fiorin, G.; Fleischmann, S.; Gorelov, S.; Gouaillardet, G.; Grey, A.; Irrgang, M. E.; Jalalpour, F.; Jordan, J.; Kutzner, C.; Lemkul, J. A.; Lundborg, M.; Merz, P.; Miletic, V.; Morozov, D.; Nabet, J.; Pall, S.; Pasquabisceglie, A.; Pellegrino, M.; Santuz, H.; Schulz, R.; Shugaeva, T.; Shvetsov, A.; Villa, A.; Wingbermuehle, S.; Hess, B.; Lindahl, E. *GROMACS 2024.0 Manual*; **2024**.
- (66) Essmann, U.; Perera, L.; Berkowitz, M. L.; Darden, T.; Lee, H.; Pedersen, L. G. A Smooth Particle Mesh Ewald Method. *J. Chem. Phys.* **1995**, *103* (19), 8577–8593.
- (67) York, D. M.; Darden, T. A.; Pedersen, L. G. The Effect of Long-Range Electrostatic Interactions in Simulations of Macromolecular Crystals: A Comparison of the Ewald and Truncated List Methods. *J. Chem. Phys.* **1993**, *99* (10), 8345–8348.
- (68) Bernetti, M.; Bussi, G. Pressure Control Using Stochastic Cell Rescaling. *J. Chem. Phys.* **2020**, *153* (11), 114107.
- (69) Humphrey, W.; Dalke, A.; Schulten, K. VMD: Visual Molecular Dynamics. *J. Mol. Graph.* **1996**, *14* (1), 33–38.
- (70) Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera—A Visualization System for Exploratory Research and Analysis. *J. Comput. Chem.* **2004**, *25* (13), 1605–1612.
- (71) Amendola, V.; Meneghetti, M. Controlled Size Manipulation of Free Gold Nanoparticles by Laser Irradiation and Their Facile Bioconjugation. *J. Mater. Chem.* **2007**, *17* (44), 4705.
- (72) Litti, L.; Meneghetti, M. Predictions on the SERS Enhancement Factor of Gold Nanosphere Aggregate Samples. *Phys. Chem. Chem. Phys.* **2019**, *21* (28), 15515–15522.
- (73) Yin, H.; Zhou, X.; Huang, Y.-H.; King, G. J.; Collins, B. M.; Gao, Y.; Craik, D. J.; Wang, C. K. Rational Design of Potent Peptide Inhibitors of the PD-1:PD-L1 Interaction for Cancer Immunotherapy. *J. Am. Chem. Soc.* **2021**, *143* (44), 18536–18547.
- (74) Maute, R. L.; Gordon, S. R.; Mayer, A. T.; McCracken, M. N.; Natarajan, A.; Ring, N. G.; Kimura, R.; Tsai, J. M.; Manglik, A.; Kruse, A. C.; Gambhir, S. S.; Weissman, I. L.; Ring, A. M. Engineering High-Affinity PD-1 Variants for Optimized Immunotherapy and Immuno-PET Imaging. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112* (47), E6506–E6514.
- (75) Pascolutti, R.; Sun, X.; Kao, J.; Maute, R. L.; Ring, A. M.; Bowman, G. R.; Kruse, A. C. Structure and Dynamics of PD-L1 and an Ultra-High-Affinity PD-1 Receptor Mutant. *Structure* **2016**, *24* (10), 1719–1728.
- (76) Tseng, T.-S.; Lee, C.-C.; Chen, P.-J.; Lin, C.-Y.; Chen, W.-C.; Lee, Y.-C.; Lin, J.-H.; Chen, K.-W.; Tsai, K.-C. Structure-Guided Discovery of PD-1/PD-L1 Interaction Inhibitors: Peptide Design, Screening, and Optimization via Computation-Aided Phage Display Engineering. *J. Chem. Inf. Model.* **2024**, *64* (5), 1615–1627.
- (77) Zhu, X.; Wei, Y.; Zhang, M.; Liu, K.; Liu, Z.; Niu, Q. Development of Novel Short Peptide Inhibitor Targeted to Immune Checkpoint PD-1 LBD. *Int. J. Pept. Res. Ther.* **2025**, *31*, 48.