



Structural analysis of nanobody interactions with their prostate-specific membrane antigen binding epitopes

Gal Alon-Zchut^a, Ran Zalk^b, Truc T. Huynh^c, Michael R. Zalutsky^c, Yossi Weizmann^{b,d,e}, Raz Zarivach^{b,f,g}, Niv Papo^{a,g,*}

^a Avram and Stella Goldstein-Goren Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

^b Ilse Katz Institute for Nanoscale Science & Technology, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

^c Department of Radiology, Duke University Medical Center, Durham, NC 27710, USA

^d Department of Chemistry, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

^e Goldman Sonnenfeldt School of Sustainability and Climate Change, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

^f Department of Life Sciences, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

^g National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

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ABSTRACT

Prostate-specific membrane antigen (PSMA), overexpressed in prostate cancer, is a promising target for diagnostics and therapy. However, the monoclonal antibodies in current use for PSMA targeting and inhibition have suboptimal activities due to their poor tissue and cell penetration and slow normal tissue clearance. Potentially superior alternatives are nanobodies (NBs), the single-chain variable domains of heavy-chain antibodies derived from camelids. The advantages of NBs include small size (~15 kDa), ability to bind hidden epitopes, and rapid clearance. In contrast to most known PSMA inhibitors, which bind to the same catalytic site in PSMA, NBs can bind to different PSMA epitopes, facilitating heterovalent binding strategies that could enhance their therapeutic and diagnostic potential. The objective of this study was to map these binding epitopes and hence to acquire an atomic-resolution understanding of NB-PSMA binding by investigating the structural interactions between PSMA and three NBs (NB7, NB8, and NB37). Using cryo-electron microscopy to generate high-resolution structures of NB-PSMA complexes, we found that NB7 had the highest affinity for PSMA due to a larger interface and to stabilizing interactions, including salt bridges and π - π stacking. Notably, we also found that NB7 and NB8 can bind simultaneously to different PSMA epitopes without interfering with the function of PSMA (which is still not completely known), opening the way for the development of theranostic applications for prostate cancer treatment and imaging. Importantly, NB7 binds specifically to human PSMA but not to murine PSMA, due to key amino acid differences responsible for its species specificity.

Classification

Major category: Biological sciences

Minor category: Biochemistry

1. Introduction

Prostate cancer is the fourth most common cancer worldwide, the second most common cancer diagnosed in men, and the eighth leading cause of cancer deaths globally—as of 2022, the latest statistics available [1]. A great deal of work has thus been devoted to unraveling the

molecular characteristics of prostate cancer, and those studies have led to significant advances in diagnostic and therapeutic strategies. Particular emphasis has been placed on prostate-specific membrane antigen (PSMA), which is overexpressed in prostate cancer and has been exploited both as a diagnostic marker for imaging and as a therapeutic target [2,3]. The exact biological roles of PSMA are not fully known, but – given that PSMA ligands are taken up via endocytosis – it is believed that PSMA has a transport function [4]. PSMA also has enzymatic activity in cleaving α -linked glutamate from *N*-acetyl aspartyl glutamate and γ -linked glutamates from polyglutamated folates, which play a role in folate metabolism [5]. PSMA is a type II transmembrane glycoprotein

* Corresponding author at: Avram and Stella Goldstein-Goren Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel.

E-mail address: papo@bgu.ac.il (N. Papo).

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composed of 750 amino acids (AAs), of which 19 comprise a small intercellular domain, 24 make up a transmembrane domain, and 707 constitute a large extracellular domain that, in turn, has three distinct domains—the protease, apical and helical domains (*SI Appendix*, Fig. S1A). The protease domain (residues 57–116 and 352–590) includes a binuclear zinc site, catalytic residues, and an arginine patch that is believed to be involved in substrate binding. The two Zn atoms, which are crucial for the enzymatic activity of PSMA, are coordinated to the AAs of the apical and protease domains [6]. The helical domain (residues 592–750) plays an important role in creating the catalytically active dimeric form of PSMA [7], which is the native conformation displayed on prostate cancer cells. Importantly, the PSMA homodimer exhibits elevated carboxypeptidase activity [8], which has been linked to prostate cancer progression [9–11]. The apical domain (residues 117–351), which is involved in ligand binding and recognition, contains the binding site for the monoclonal antibody (mAb) J591—the first mAb shown to target the extracellular domain of PSMA [12]. Since its discovery in the 1990s, J591 has been the subject of numerous studies focusing on PSMA-targeted mAb-drug conjugates [13–17] and radio-labeled mAbs [18–22]; nonetheless, the structure of PSMA bound to J591 is not available.

Most proteins currently used as PSMA-targeting theranostic agents are mAbs such as J591, but these molecules have several limitations, particularly poor penetration into tumor tissue and suboptimal drug-delivery efficiency [23]. A promising alternative approach is the use of nanobodies (NBs), the single-chain variable domains of heavy-chain antibodies derived from camelids. NBs are small fragments (~15 kDa) with high affinity and specificity for a particular target [24]. In comparison to Abs, NBs exhibit better tissue penetration and have the added advantage of being able to target epitopes that are not readily accessible [25].

In our previous studies [26,27], four NBs with various affinities toward PSMA were identified and partially characterized, including the resolution of the crystal structures of three of the four (NB7, PDB ID 6XXN; NB8, PDB ID 6XXO; and NB37, PDB ID 6XXP). The highest affinity NB, i.e., NB7, was conjugated to doxorubicin, with the conjugate demonstrating rapid internalization (within minutes) and drug release into PSMA⁺ cells, resulting in excellent cytotoxic activity [26]. In-vivo studies in mice demonstrated that the NB-doxorubicin conjugate inhibited tumor growth as effectively as commercial doxorubicin but at a 42-fold lower dose, thereby highlighting the potential of NBs for targeted cancer therapy. It was also demonstrated that the activity of PSMA was not impaired by its binding to the above three NBs (K_d values of 0.055 nM, 6.0 nM, and 3.4 nM for NB7, NB8, and NB37, respectively) [26]. More recently [27], NB7 labeled with radioiodine or ²¹¹At demonstrated high affinity and specific binding to PSMA⁺ cells, with rapid PSMA⁺ tumor accumulation in mice and rapid clearance from non-target tissues, including the kidneys. Importantly, previous studies have shown that NB7 contains two disulfide bonds, unlike most other NBs, including NB8 and NB37, that contain only one. The ‘extra’ disulfide bond is present in the third complementarity-determining region (CDR3), probably restricting its flexibility and hence leading to the stronger NB-PSMA interaction observed for NB7 [28,29]. The extra disulfide bond influences the conformational stability of the NB, likely reducing its tendency to aggregate due to increased kinetic stability [30].

In previous work, several attempts to crystalize PSMA-NB complexes were made, however crystals of NB alone were constantly obtained. The high conformational stability of unbound NBs, including NB7, might account for the lack of success in crystallizing NB-PSMA complexes.

Since NB7, NB8 and NB37 showed promise as theranostic agents for prostate cancer, we sought to characterize the structure of those NBs in complex with PSMA as a means to better understand their binding interactions with PSMA, including differences in their affinities for PSMA. Indeed, determining the structures of such complexes is crucial for the rational design and optimization of clinically relevant NB-drug and NB-

radioisotope conjugates that specifically target PSMA. Additionally, knowledge of the binding sites of NBs to PSMA can be leveraged for two purposes: ensuring that the NB does not interfere with the active site of PSMA (i.e., its physiological functions) and utilizing the NBs in combination strategies (multiple NBs binding to different epitopes or NBs administered in tandem with PSMA inhibitors such as Pluvicto, an FDA-approved drug that targets the active site of PSMA [31] that provide increased potency [32] and contribute to preventing drug resistance [33].

Since our previous attempts to crystalize PSMA-NB complexes yielded only crystals of NB alone (possibly due to the high conformational stability of unbound NBs), we took a different approach to solve the structures of NB-PSMA complexes in the current study: we used single-particle cryogenic electron-microscopy (cryo-EM) to solve the structures of three different NB-PSMA complexes at an average resolution of 2.61 Å. For each complex, we showed that two NBs bind to one PSMA dimer and that none of the three NBs binds near the PSMA catalytic site. Our structural results indicate that the dissimilarities in the binding affinities of the three NBs for PSMA result from different epitope-binding areas for each NB and differences in the interactions between the NBs and PSMA. We also showed that NB7 and NB8 can bind simultaneously to PSMA, a finding that can be potentially utilized for developing combination therapies.

2. Results

2.1. NB-PSMA assembly and structure determination

To elucidate the differences in affinities and to identify the specific sites of interactions at atomic resolution, the three NBs (NB7, NB8 and NB37) were expressed and purified from *Escherichia coli* strain WK6 bacteria, according to established protocols [26]. The purified extracellular domain of human PSMA (hPSMA) [residues 44–750 [7]; purchased from Caltech Protein Expression Center, CA, USA] was separated into monomeric and dimeric forms (*SI Appendix*, Fig. S2), where the form was controlled by buffer exchange [6]. Since hPSMA exists as a symmetrical homodimer in vivo [8], the dimer was chosen for creation of the NB complexes [6]. hPSMA was incubated with NB7, NB8, or NB37 in 1:10, 1:6, or 1:3 M ratios, respectively. The relevant complex was then separated from free NB by size-exclusion chromatography (SEC) (Fig. 1A), and the samples were analyzed using 12.5 % SDS-PAGE and 12 % native PAGE (Fig. 1B and C and *SI Appendix*, Fig. S3). Comparison of the SDS-PAGE and native PAGE gels showed distinct bands of hPSMA and NB in the SDS-PAGE gel, indicating the presence of both components within the sample. In addition, a single band was observed in the native PAGE gel, strongly suggesting the successful creation of an NB-PSMA complex.

Subsequently, the structures of the complexes were determined using single-particle cryo-EM, as outlined in the workflow presented in *SI Appendix*, Fig. S4. Thereafter, atomic models of the NB7-PSMA (PDB ID 9HLW), NB8-PSMA (PDB ID 9HVI) and NB37-PSMA (PDB ID 9HVL) complexes at final resolutions of 2.7 Å, 2.4 Å, and 2.7 Å, respectively [Fourier shell correlation (FSC) = 0.143], were built manually based on cryo-EM 3D reconstruction maps (*SI Appendix*, Fig. S5–S8). The structural analyses revealed that two NBs bind to a single hPSMA dimer. Specifically, NB7 was found to bind to the helical domain, and NB8 and NB37, to the apical domain (Fig. 1D–I). Notably, as mentioned above, the binding sites for all three NBs were not near the hPSMA active site located within the protease domain, and none interfered with the activity of hPSMA [26].

2.2. Analysis of specific interactions in NB-PSMA complexes

Our next step aimed to verify the superior affinity of NB7 for hPSMA vs. the affinities of NB8 and NB37 and to identify the NB binding sites for the three NBs. Examination of the atomic models revealed that the

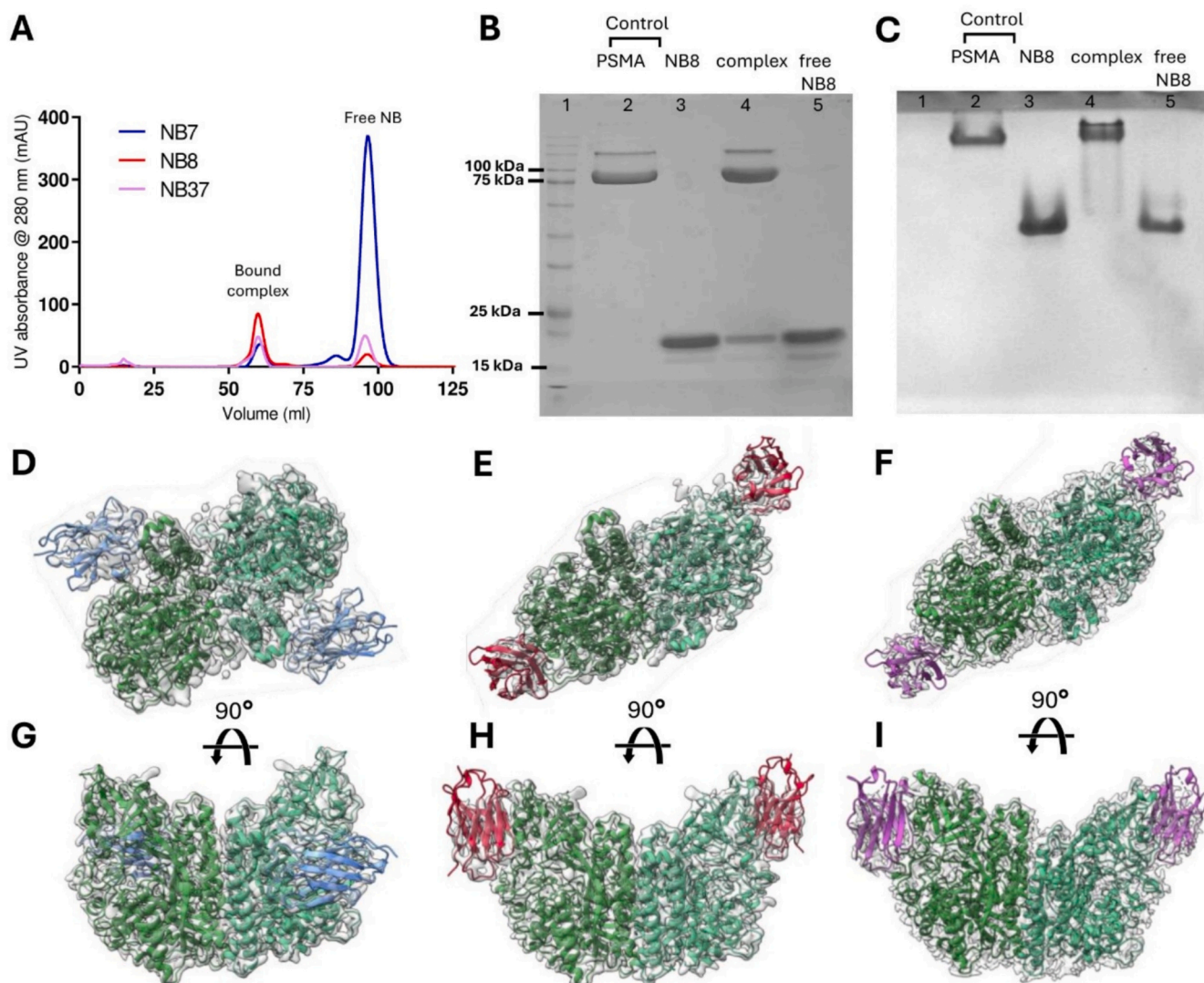


Fig. 1. Analysis of NB-PSMA complexes. (A) Size-exclusion chromatograms (SECs) showing distinct peaks for PSMA complexed with NB7, NB8, and NB37; the peaks for free NBs are also evident. (B) Protein migration patterns for the PSMA-NB8 complex under non-reducing denaturing conditions on SDS-PAGE. Lanes 1, 2, and 3 show the protein molecular weight marker, the PSMA control, and the NB8 control, respectively. Lane 4 shows the first peak from the chromatogram, confirming the presence of PSMA and NB8. Lane 5 shows the second peak from the chromatogram, indicating the presence of unbound NB8. Similar results were obtained for the other NB complexes (*SI Appendix, Fig. S3*). (C) Protein migration patterns for the PSMA-NB8 complex on native PAGE. (D-F) 3D cryo-EM maps and fitted models of PSMA (with the two monomers of the dimer shown in different shades of green) complexed with NB7 (light blue), NB8 (red) and NB37 (purple), respectively. (G-I) Side views (rotated 90°) of the above-mentioned complexes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

specific interactions between NB7 and hPSMA involved two salt bridges (*Fig. 2A* and *SI Appendix, Fig. S9A*) in comparison to only one salt bridge in the dimeric complex of NB8 with hPSMA (*SI Appendix, Fig. S9B*). In addition, the NB7-PSMA complex exhibited a notable π - π stacking interaction between Phe 633 of hPSMA and His 102 of NB7 (*Fig. 2B*), which contributes to the stabilization of the complex, through weak non-covalent forces, and enhances the overall stability of the protein-protein interface. In contrast, the structure of the NB8-PSMA complex exhibited a contiguous β -sheet made up of the β -sheets of the component proteins (two NBs and the hPSMA dimer) and extending across them (*SI Appendix, Fig. S9C*). This type of interaction – the β -sheet extension – may be viewed as structural complementation, which also stabilizes the protein-protein interface. The NB37-PSMA complex exhibited the same interactions as the NB8-PSMA complex (*SI Appendix, Fig. S10*), which was to be expected, since the sequences of NB8 and NB37 differ only by a single AA in position 13. Therefore, we chose to focus our further research on NB7 and NB8.

An analysis of the electrostatic interactions between the two proteins in the NB7-PSMA dimeric complex (*Fig. 2C*), in comparison with those in the NB8-PSMA dimeric complex (*Fig. 3A*), revealed a distinct difference between the two complexes, with the NB7-PSMA complex exhibiting a greater number of electrostatic interactions. In contrast, an analysis of the hydrophobic interactions revealed minimal differences between the two complexes (*Fig. 2D* and *Fig. 3B*). These analyses of the different types of interaction taking place in the complexes proffer an explanation for the higher affinity of NB7 vs. NB8 and NB37 to hPSMA, which lies in the larger interface area, the extra salt bridge, the higher number of electrostatic bonds, and the π - π interactions, as evaluated by using the PDBePISA (Proteins, Interfaces, Structures and Assemblies) tool (*Table 1*).

2.3. Simultaneous binding of NB7 and NB8 to hPSMA

The observation of the distinct and distant binding sites of NB7 and

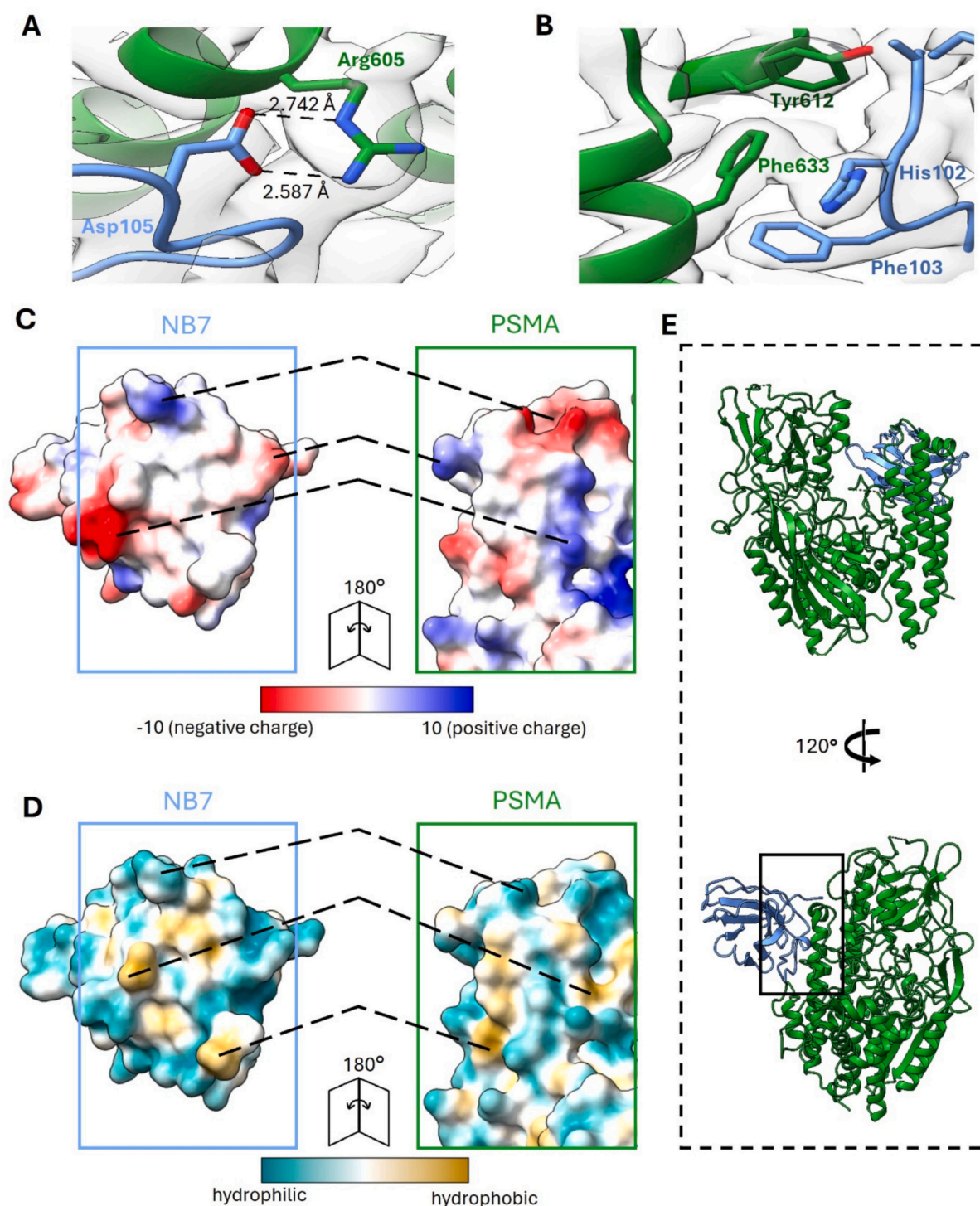


Fig. 2. Detailed interactions between PSMA and NB7 within the PSMA-NB7 complex. (A) Depiction of a salt bridge between Arg 605 on PSMA and Asp 105 on NB7, contributing to the stabilization of the complex. (B) Illustration of π - π stacking interactions, which significantly enhance binding affinity. Electron density mapping reveals a well-defined channel between Phe 633 on PSMA and His 102 on NB7. (C) Visualization of the electrostatic surface potential of the interacting areas on NB7 (left) and PSMA (right); colour scale bar: red, regions of negative potential (electron-rich areas); white, neutral areas; blue, regions of positive potential; values range from -10 (blue) through 0 (white) to 10 (red). Dotted lines indicate specific interaction sites. (D) Hydrophobicity of the interacting surface areas of the two proteins, ranging from hydrophilic (blue) to hydrophobic (gold). (E) Structural overview of a PSMA monomer (green) and an NB7 molecule (blue), with the interacting surface area of the proteins enclosed in a black rectangle. The total interaction surface area is approximately $830 \text{ \AA}^2$, highlighting the relatively large contact region [59]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NB8 to hPSMA motivated us to explore whether the two NBs can bind simultaneously to hPSMA. To this end, a series of experiments aimed at forming a new tri-protein complex were performed. Initially, an SPR dual-binding experiment was conducted in which NB7 was immobilized

on a chip, and its baseline level was determined (Fig. 4A). This step was followed by saturation of NB7 with hPSMA, after which NB8 was injected. The experiment demonstrated that NB8 successfully bound to hPSMA that was already bound to NB7, indicating two distinct hPSMA

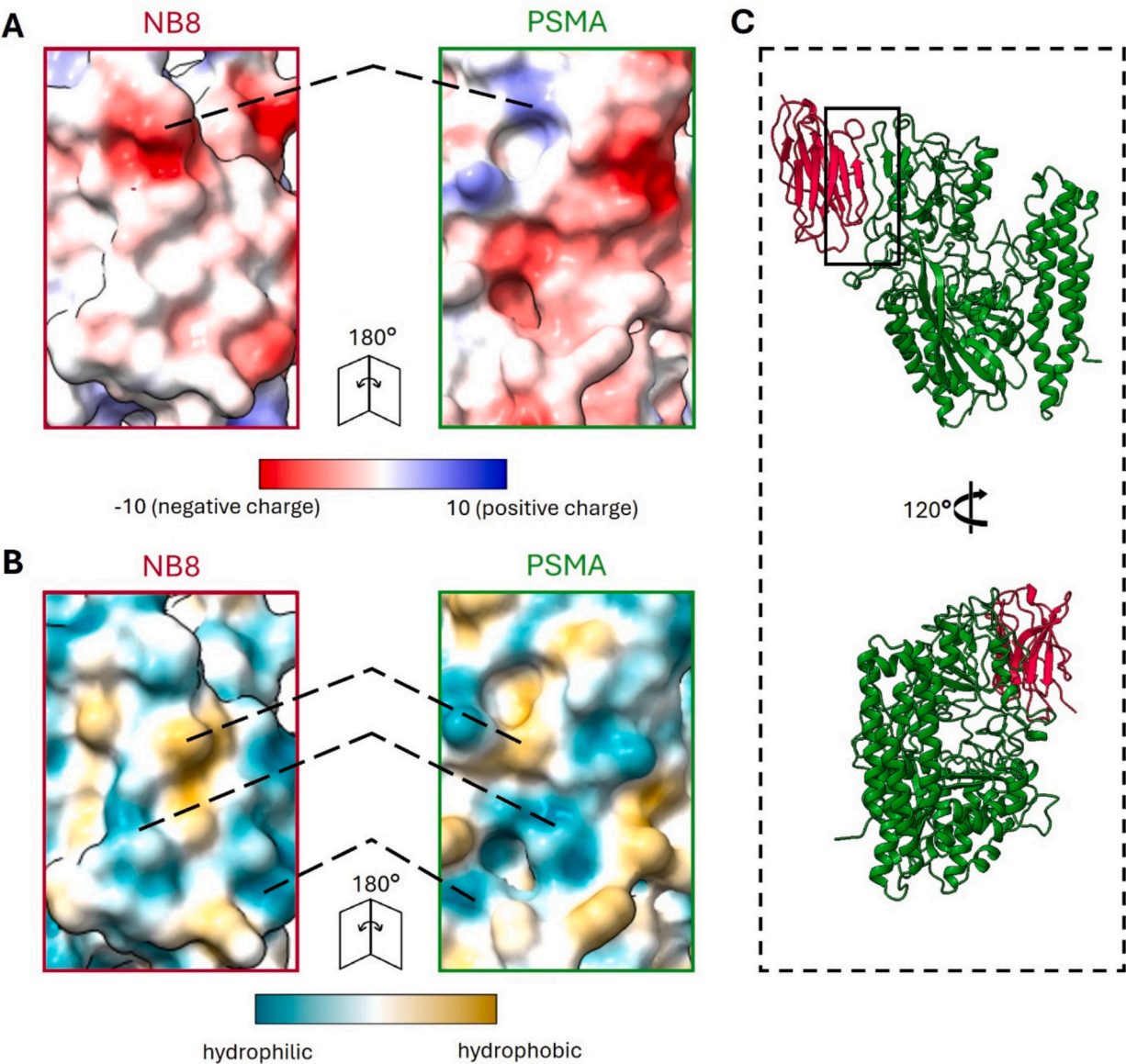


Fig. 3. Electrostatic and hydrophobic surface interactions between PSMA and NB8. (A) The electrostatic surface potential of the interacting areas on NB8 (left) and PSMA (right), colour-coded from negative charge (red) to positive charge (blue). Dotted lines indicate specific interaction sites. (B) Hydrophobic surface representation of the same interacting surface areas, colour-coded from hydrophilic (blue) to hydrophobic (gold). (C) Structural overview of a PSMA monomer (green) and an NB8 molecule (red) with the interacting surface area of the proteins enclosed in a black rectangle. The total interaction surface area is approximately 670 Å. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
PDBePISA analysis of the interfaces in the different NB-PSMA complexes.

	NB7-PSMA	NB8-PSMA	NB37-PSMA	NB7-PSMA in NB7-PSMA-NB8	NB8-PSMA in NB7-PSMA-NB8
Average interface area (Å ²)	828.7 ± 10.5	676.8 ± 8.0	690.7 ± 2.4	836.3 ± 4.1	710.9 ± 8.7
Number of hydrogen bonds	12	12	12	10	10
Number of salt bridges	2	1	1	2	0
Number of electrostatic bonds	3	1	1	3	1
Number of π - π interactions	1	0	0	1	0

binding sites for the two NBs and the ability of hPSMA to simultaneously bind NB7 and NB8 (Fig. 4B). A control experiment was conducted to exclude heterodimer formation, i.e., to show that NB8 does not interact with NB7, as was indeed confirmed (Fig. 4B). To further validate these findings, an additional SPR experiment was performed in which NB8 was immobilized on the sensor chip, followed by sequential injections of hPSMA and then NB7. The results, presented in *SI Appendix*, Fig. S11,

mirrored those of the initial setup, confirming the reproducibility of the dual-binding interaction. These findings further support the absence of allosteric effects or steric hindrance during dual binding of the NBs to PSMA. Subsequently, hPSMA was incubated with the two NBs in an hPSMA:NB7:NB8 ratio of 1:5.6:5.6. The resulting complex was subjected to SEC (Fig. 4C), and the peaks were analyzed using SDS-PAGE and native PAGE (Fig. 4D and E). Both gels displayed patterns consistent

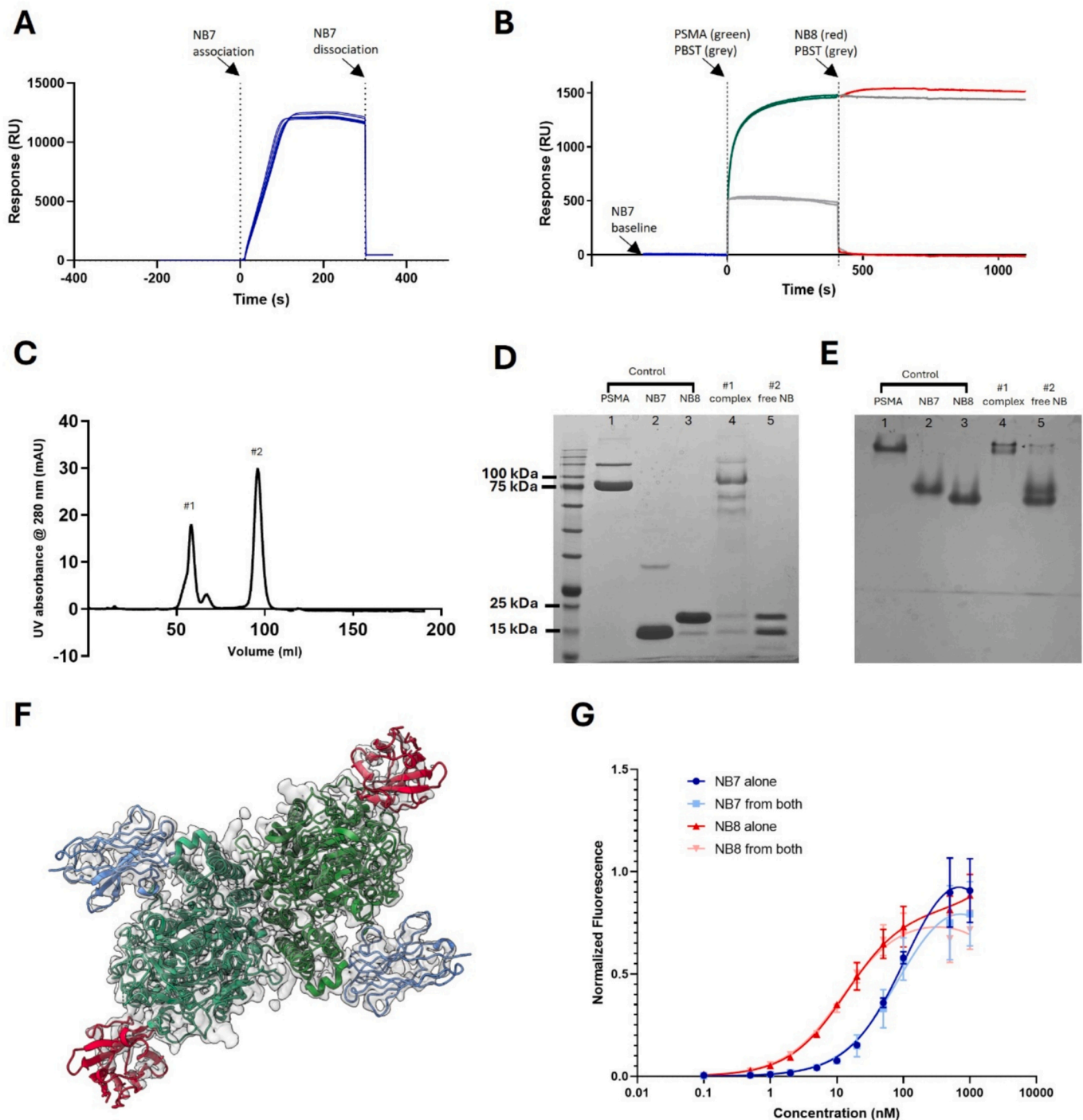


Fig. 4. Characterization of the trimeric complex of PSMA with NB7 and NB8. (A) Surface plasmon resonance (SPR) sensorgram of NB7 binding to the SPR chip. (B) SPR dual-binding experiment demonstrating concurrent binding of NB7 and NB8 to PSMA and confirming that NB8 does not interact with NB7, thereby excluding heterodimer formation. (C) Size-exclusion chromatography (SEC) profile. The first peak corresponds to the alleged complex, and the second peak corresponds to the unbound NBs. (D) SDS-PAGE analysis of SEC fractions confirms the presence of the separate constituents of the complex. (E) Native PAGE of the same fractions confirming the integrity of the complex. (F) Structural depiction of the trimeric PSMA (green) complex with NB7 (light blue) and NB8 (red). The map resolution is 3.1 Å. (G) FACS analysis ($n = 3$), assessing the binding dynamics of NB7 and NB8 (concentration range: 0.1–1000 nM) to PC3-PIP (PSMA⁺) cells revealed unimpeded dual binding. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with those observed in the formation of complexes between hPSMA and each NB, affirming the integrity of the complex. Single particle cryo-EM analysis of the complex was then performed, and its 3D structure was reconstituted at a resolution of 3.1 Å.

The NB7-PSMA-NB8 structure (PDB ID 9HVK, Fig. 4F) clearly showed that both NBs bind at the same two locations within the trimeric

complex (NB7-PSMA-NB8) as they had done in the NB7-PSMA and NB8-PSMA complexes. No major conformational changes of hPSMA were noted in the trimeric complex compared to the dimeric complexes. An analysis – using the PDBePISA tool – of the interactions between each NB and PSMA in the trimeric complex revealed an average interface area of 836.3 Å² for NB7-PSMA and 710.95 Å² for NB8-PSMA. The interface

area of NB7-PSMA in the trimeric complex was not markedly different from that calculated for the dimeric NB7-PSMA complex, and the two salt bridges were conserved in the interface area of the trimeric complex. In contrast, the interface area of NB8-PSMA in the trimeric complex was slightly higher than that in the dimeric NB8-PSMA complex, and the single salt bridge disappeared.

To assess whether the subtle changes in the interacting surface areas between hPSMA and the NBs in the relevant dimeric protein complexes vs. the trimeric protein complex influenced the binding affinity of the NBs to hPSMA, a flow cytometry experiment was conducted using PC3 PIP PSMA⁺ cells. NB7 labeled with AF-647 and NB8 labeled with DyLight™ 488 were incubated with the cells at different concentrations, either individually or in combination. As shown in Fig. 4G, there are no significant differences in the binding affinities of NB7 and NB8 for hPSMA when incubated alone or together at equivalent concentrations, indicating that the one NB does not interfere with the binding of the other NB to hPSMA. Although greater variability was obtained at higher NB concentrations, the overall binding affinities remained comparable regardless of whether the NBs were tested individually or in combination.

2.4. NB7 binding to human PSMA vs. murine PSMA

NB7 was chosen for this part of the study by virtue of its superior affinity for hPSMA vs. NB8 and NB37, making it the most promising candidate for drug development. To evaluate the potential of NB7 for in-vivo applications, it was first necessary to assess the cross-species reactivity of NB7 to enable proper interpretation of preclinical studies in xenografted murine models and the future development of NB7 as a theranostic agent. To this end, the binding affinities of NB7 to both hPSMA and murine PSMA (mPSMA) were investigated. An SPR experiment to determine the specific binding of NB7 to hPSMA gave an affinity constant (K_d) of 0.0359 ± 0.0007 nM, an association rate constant (k_{on}) of $1.67 \pm 0.03 \times 10^6$ 1/M 1/s, and a dissociation rate constant (k_{off}) of

$6.01 \pm 0.05 \times 10^{-5}$ 1/s, with the K_d value indicating a high-affinity interaction. In contrast, no specific binding of NB7 to mPSMA was detected (SI Appendix, Fig. S12), despite an 87 % overall sequence similarity between the two proteins (Fig. 5A).

With the structure of the NB7-PSMA complex in hand, we could compare the NB7-interacting interfaces of hPSMA and mPSMA as a means to understanding the exquisite selectivity of NB7 for hPSMA. Since the structure of mPSMA had never been determined, we based our analysis on an AlphaFold prediction [34] of a monomer (UniProt O35409). Overlay of the hPSMA and mPSMA structures gave a root mean square deviation (RMSD) value of 1.104 Å, indicating highly similar structures, as is often seen between homologous proteins. Of the 17 residues at the NB7-PSMA interface, there was a visible difference in three AAs between hPSMA and mPSMA, as follows. The difference between Arg 605 in hPSMA and Lys 607 in mPSMA is relatively inconsequential due to the similar positive charge and electron density of these two residues (Fig. 5B). The replacement of Lys 610 in hPSMA by Thr 612 in mPSMA results in a loss of interaction between PSMA and NB7 (Fig. 5C). The difference between Ser 613 in hPSMA and Asn 615 in mPSMA causes a significant steric clash between Asn 615 on mPSMA and the residue in position 100 on NB7 (Fig. 5D), constituting a potential steric barrier blocking NB7 from binding to the target protein. It is these latter two differences that probably disrupt NB7-mPSMA interactions.

It is evident that despite the overall high sequence similarity, differences in specific amino acids in the NB7-PSMA interaction region impact the binding dynamics and stability of the NB7-hPSMA and NB7-mPSMA complexes. This specificity stands in contrast to the significant cross-reactivity of small-molecule inhibitors targeting the active site in human and murine PSMA [35].

3. Discussion

Numerous structures of hPSMA bound to small-molecule inhibitors [36–38] or to the A9g RNA aptamer [39] have been solved using X-ray

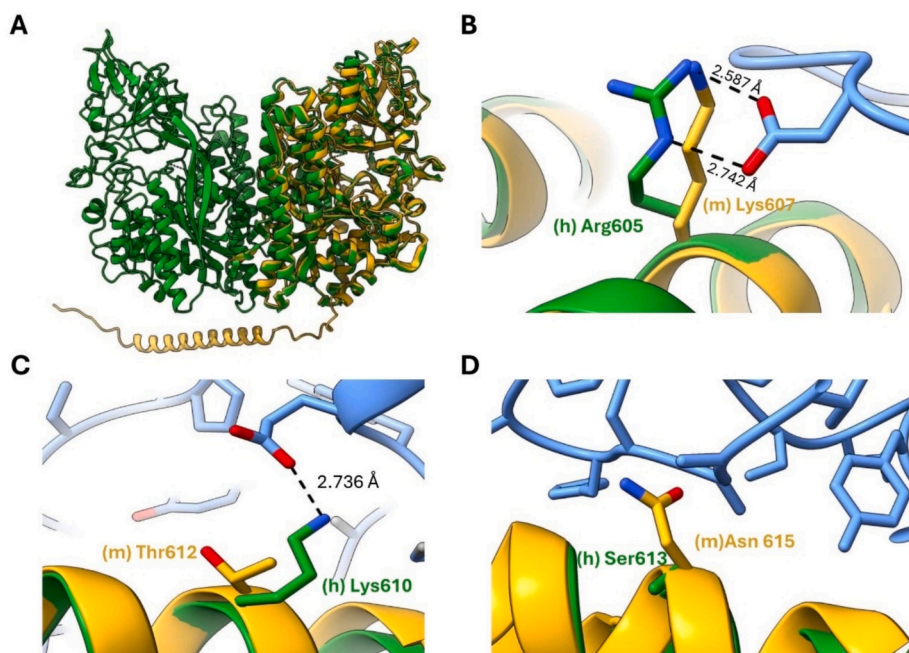


Fig. 5. Structural comparison of murine PSMA (mPSMA) and human PSMA (hPSMA). (A) Overlay of the dimeric hPSMA (green) and monomeric mPSMA (yellow) structures. The murine model is based on an AlphaFold prediction of the monomer (UniProt O35409). (B–D) Specific amino acid differences between hPSMA and mPSMA, as follows: (B) The lysine substitution in mPSMA shows minimal structural influence. (C) The presence of a threonine residue in mPSMA means that this protein has only one salt bridge vs. two salt bridges in hPSMA, which probably affects its interaction with NB7. (D) Replacement of serine in hPSMA with asparagine in mPSMA may destabilize the NB-PSMA interaction due to the greater bulkiness of the side chain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

crystallography. Nonetheless, this structural tool has three main drawbacks that can impede further research on hPSMA inhibitors: i) the method requires large amounts of pure protein samples; ii) the crystallization process can alter the protein structure, and iii) X-ray crystallography can provide atomic structure resolution for proteins of various sizes, but only if the protein of interest can be crystallized; for example, our previous attempts to obtain the crystal structures of hPSMA bound to different NBs were unsuccessful, yielding crystals of NB alone. This could be due to the high stability of the NBs, which causes them to crystallize rapidly without forming crystals of the complex and the overall high stability of the formed NB crystal. Cryo-EM imaging can overcome the above three limitations—particularly, the preservation of the native state of proteins. Maintaining the sample in a state that more closely resembles its conformation and interactions in its native environment is crucial for gaining deeper insights into biological function [40]. In the current study, the high-resolution cryo-EM structures of the NB-PSMA complexes provided detailed insight into their binding interactions at the atomic level, in that differences between NB7, NB8 and NB37 in electrostatic interactions, hydrogen bonding, and π - π interactions could explain their different affinities for hPSMA and their therapeutic and/or diagnostics potential. In a previous study [26], the affinities of NB7, NB8, and NB37 for hPSMA were determined to be 0.055 nM, 6.0 nM, and 3.4 nM, respectively, i.e., the affinity of NB7 for hPSMA was two orders of magnitude higher than that of NB8 and NB37. In light of the findings of the current study, we can now attribute these differences to the larger numbers of salt bridges (2 in the NB7-PSMA complex vs. 1 in the other two complexes), of electrostatic bonds (3 vs. 1), and of π - π interactions (1 vs. 0), as well as the larger overall interface area of the NB7-PSMA complex ($828.7 \pm 10.5 \text{ \AA}^2$) vs. the other two complexes ($676.87 \pm 8.0 \text{ \AA}^2$ for PSMA-NB8 and $690.7 \pm 2.4 \text{ \AA}^2$ for PSMA-NB37). Understanding how these structural features influence binding affinities is important for both drug development and for the use of NBs as diagnostic and therapeutic agents. For example, it was previously reported that signal clearance in mice injected with fluorescently labeled NBs required longer times for NBs with higher affinities for PSMA (24 h for NB37, 32 h for NB8, and > 56 h for NB7) [26].

One of the most important outcomes of the current study is the resolution of the novel structure of NB7 and NB8 simultaneously bound to hPSMA. To the best of our knowledge, this is the first study presenting the structural determination of the precise binding sites of two different NBs (or mAbs) binding simultaneously to two distinct epitopes on a single target: NB7 binds to the protease and helical domains of hPSMA, while NB8 binds to its apical domain. Neither of the NBs binds near the catalytic site of hPSMA (*SI Appendix*, Fig. S1B and C), and thus they do not interfere with its function (which is still not fully known), as supported by our previous study [26]. Thus, NB7 and NB8 do not disrupt the activity of hPSMA, which should hence be preserved upon binding. These findings are in direct contrast to the activity of hPSMA inhibitor PSMA-617, which binds to the active site (PDB ID 8BOW) [22]. The dual binding of NB7 and NB8 offers a unique therapeutic advantage in that it can be exploited for combinational therapy, where each NB would be conjugated to a different drug or labeled with a different radionuclide, thus potentially increasing treatment efficacy. Alternatively, the dual binding could be leveraged to engineer a hybrid molecule incorporating one or both NBs and/or a hPSMA inhibitor, thereby enabling multiple-site binding, which may enhance overall binding affinity. The distance between the C terminal of NB7 and the N terminal of NB8 is about 65 Å in the trimeric complex (NB7-PSMA-NB8), hence when choosing a linker for fusing NB7 with NB8, its length should be >65 Å. A linker between NB7 or NB8 and a hPSMA inhibitor should be of length ~ 60 Å or 40 Å, respectively. Possible linkers that could be used are Gly/Ser (i.e., four repeats of Gly₄Ser to fuse NB7 with NB8, or three Gly₄Ser repeats to fuse NB8 with a small molecule hPSMA inhibitor) [41] or chemical linkers such as polyethylene glycol (PEG) (i.e., PEG of 1 kDa to fuse NB7 to NB8, or < 1 kDa for fusing the NBs to small molecule hPSMA inhibitors) [42]. Moreover, if mutations should occur in the hPSMA extracellular domain,

the ability to target different regions of hPSMA could circumvent this potential resistance mechanism.

A close examination of the small differences in the characteristics of NB7-PSMA, NB8-PSMA and NB7-PSMA-NB8 revealed that the interaction surface area of NB7-PSMA was not significantly different from that in the trimeric NB7-PSMA-NB8 complex ($828.7 \pm 10.5 \text{ \AA}^2$ vs. $836.3 \pm 4.1 \text{ \AA}^2$), despite the loss of two hydrogen bonds in NB7-PSMA-NB8 (*Table 1*)—a finding that is in keeping with a previous report that certain interactions in protein-protein binding can compensate for the loss of others, thereby maintaining the overall binding affinity [43]. In contrast, the interaction surface area of NB8-PSMA was slightly smaller than that of NB7-PSMA-NB8 ($676.8 \pm 8.0 \text{ \AA}^2$ vs. $710.9 \pm 8.7 \text{ \AA}^2$), but with the loss of the salt bridge. The slight increase in the surface area of the NB8-PSMA interface in the NB7-PSMA-NB8 complex could be caused by a shift in the orientation or position of the residues at the interface, leading to a rearrangement that interrupts the formation of the salt bridge. Such modifications can be induced by subtle changes in the protein conformation or the binding dynamics of the NB, which may not always manifest as major overall structural changes [44]. This notion is supported by our *in vitro* experiments (*Fig. 4F*), which showed that there were no major changes in the binding capabilities of NB7 and NB8 to hPSMA-overexpressing cells when added either alone (PSMA-NB7 or PSMA-NB8) or together (NB7-PSMA-NB8). Importantly, this finding supports the notion that these NBs can be used together in therapeutic or diagnostic applications without compromising their individual effectiveness. Moreover, when examining the 3D classes of the NB7-PSMA-NB8 complex, all classes showed occupancy of NB7, but a subset of minor classes exhibited partial occupancy of NB8 (*SI Appendix*, Fig. S13). This observation could be strongly associated with the differences in affinities between the two NBs toward PSMA.

An additional key structural observation made in this study derived from the comparison between the crystal structure of hPSMA (PDB ID 1Z8L) [7] and the hPSMA structures from the solved cryo-EM complexes. We note that the comparison was made with the PDB crystal structure because of our inability to determine the cryo-EM structure of PSMA alone due to its strongly preferred orientation, which led to very low resolution (*SI Appendix*, Fig. S14). Importantly, examination of the crystal contact area of hPSMA (*SI Appendix*, Fig. S15A) revealed that it is distant from the binding site of the particular NB. In addition, overlaying the crystal structure of hPSMA on the structure of hPSMA in the NB7-PSMA, NB8-PSMA, NB37-PSMA and NB7-PSMA-NB8 complexes revealed only minor differences between the structures (*SI Appendix*, Fig. S15B-E), suggesting that the binding of the NBs does not significantly alter the native structure of hPSMA, i.e., its conformational integrity is preserved.

We identified three critical sites in hPSMA that are involved in the binding to NB7, namely, Arg 605, Lys 610 and Ser 613, all located in the helical domain of PSMA. We noticed that any changes at these three specific sites, as seen in mPSMA, would probably contribute to the loss of interaction between NB7 and mPSMA, although it is possible that some such mutations play a significant role while others do not. This finding highlights the importance of structural studies on the interaction between a protein-based drug and its tumor-associated target, such as the interactions reported herein. In this case, understanding the structural rationale for species specificity in PSMA binding provides a rational path for the preclinical development of NB7 as a theranostic platform for metastatic prostate cancer.

The finding that NB7 binds with high affinity to hPSMA but not to mPSMA is particularly important when viewed against the background of the many studies investigating PSMA-targeted agents, namely, the development of small-molecule PSMA inhibitors in murine models. Most small-molecule PSMA inhibitors bind to both hPSMA and mPSMA to unpredictable degrees. As a result, murine biodistribution data can lead to erroneous conclusions about the potential for renal radiotoxicity of radiolabeled PSMA inhibitors because PSMA expression in the kidneys is much higher in mice than in humans. Furthermore, the relative binding

of various radiolabeled PSMA inhibitors to mPSMA differs from that to hPSMA, making it difficult to select the drugs that warrant clinical evaluation. For example, in mice, the radiation dose delivered to the kidneys is about 30 times higher for ^{177}Lu -PSMA-I&T than for ^{177}Lu -PSMA-617 [45], while in humans the difference is a factor of as little as ~ 1.5 [46]. The lack of reactivity of NB7 with mPSMA has two additional implications. First, therapeutic effects observed in mice can confidently be attributed to the interaction of NB7 with hPSMA on human xenograft tumor cells, rather than to off-target effects on murine tissue. And second, PSMA expression levels in normal murine tissue are generally higher than those in humans [46], with the consequence that the development of a radiopharmaceutical that would be valuable for human use might be erroneously abandoned based on its high uptake in normal murine tissue. While cryo-EM is a powerful tool for resolving large macromolecular assemblies, it faces several limitations when applied to multi-protein complexes. In the case of the NB7-PSMA-NB8 complex, we observed compositional heterogeneity, a common feature in cryo-EM datasets of multi-subunit assemblies [47–49], which may reduce the resolution and complicate the interpretation of specific regions. Indeed, this conformational heterogeneity, particularly in flexible regions of the complex, led to blurred or incomplete density maps as seen in *SI Appendix*, Fig. S13. Moreover, the lower affinity of NB8 for PSMA compared to NB7 may also contribute to dissociation during sample preparation, resulting in underrepresentation of intact trimers and limiting resolution, as seen in *SI Appendix*, Fig. S8. Finally, strong preferred orientation of PSMA alone on the EM grid hindered accurate 3D reconstruction of the unbound protein, preventing structural comparison between the unbound and complex forms.

Finally, some attention should be given to possible immunogenic effects of NBs used in therapeutic applications. Although NBs are derived from camelids and nurse sharks (VHHs and VNARs, respectively), they possess several intrinsic features that confer a relatively low immunogenicity risk in humans: their small size limits the number of potential epitopes; they exhibit a low tendency to form aggregates, which are known to enhance immunogenicity; and, in the case of non-half-life-extended NBs, their rapid clearance from circulation further minimizes immune exposure [50]. Clinical studies have indeed generally reported low immunogenicity of NBs, particularly when humanized [51]. Therefore, we believe that administration of an NB-PSMA-NB trimer, especially if the NBs are humanized, is unlikely to cause a strong immune response. Nonetheless, additional studies are warranted to fully assess the immunological implications of a therapeutic strategy of this type.

4. Conclusions

The structural insights provided by the high-resolution cryo-EM analysis of each PSMA-NB complex have deepened our understanding of the molecular interactions that direct the differing affinities of NB7, NB8, and NB37 for PSMA. The structural features of the NB7-PSMA complex (larger interaction surface area, high number of salt bridges and the presence of a π - π interaction), account for its significantly higher affinity, making it a promising candidate for developing PSMA-targeted theranostic agents. The ability of NB7 and NB8 to simultaneously bind to PSMA without compromising their individual binding capabilities offers a new avenue for combination therapies. Each NB could be conjugated to either the same imaging radionuclide or to the same or different therapeutic radionuclides, potentially enhancing efficacy. Alternatively, one NB could be used for imaging and the other, for targeted radionuclide therapy, as has been done for ^{68}Ga -PSMA-11 as a PET/CT imaging agent and ^{177}Lu -PSMA-617 as a radioligand therapy for metastatic prostate cancer [52]. Additionally, the specificity of NB7 for hPSMA, as opposed to mPSMA, underscores the importance of understanding and accounting for species-specific interactions in preclinical models, ensuring that therapeutic effects are accurately attributed to the intended target. These findings pave the way for the rational design of new

nanobodies for use alone or in heterobivalent form with improved therapeutic potential and precision.

5. Materials and methods

5.1. Purification of NBs

DNA encoding for the NBs [NB7, NB8, and NB37 [26]] was transformed into *Escherichia coli* strain WK6. The bacteria were grown in liquid LB-Ampicillin overnight at 37 °C and 200 rpm before being transferred to TB medium (16.9 mM KH_2PO_4 , 94 mM of $\text{KH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 12 g/L peptone, 24 g/L yeast extract, 0.4 % glycine, 0.1 % ampicillin, 0.1 % MgCl_2 , and 0.47 % glucose) at 37 °C and 250 rpm until an OD_{600} of 0.6–0.9 was reached. Then, induction was performed with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) (Tzamal D-Chem Laboratories, Israel), and the temperature was adjusted to 28 °C overnight, followed by periplasmic extraction with 16 mL of TES buffer (500 mM sucrose, 200 mM Tris-HCl, 0.5 mM EDTA, pH 8) for 3 h and then 16 mL of TES buffer diluted 1:4 overnight. The NBs were purified using a HisTrapTMHP 5-mL column (Cytiva, Malborough, MA, USA) in an ÄKTATM start protein purification system. The column was first equilibrated with 5 column volumes (CV) of running buffer (800 mL of PBS $\times$ 1 and 10 mM imidazole) and then the sample was loaded at a flow rate of 1 mL/min using a pump. The column was washed with 10 CV of washing buffer (400 mL of PBS $\times$ 1, 10 mM imidazole and 0.1 % Triton X-114), and a second wash was performed with 5 CV of running buffer. Lastly, the desired protein was eluted from the column with elution buffer (400 mL of PBS $\times$ 1 and 500 mM imidazole) and collected in fractions from a minimum mAU of 10 until 115 mAU was reached (average mAU of eluted imidazole). The imidazole was removed on a desalting column. The size and purity of the proteins were evaluated using 15 % SDS-PAGE gel electrophoresis, which confirmed the expected size of approximately 16 kDa. The yield of each purification process was determined using a NanoDrop spectrophotometer (Thermo Scientific, MA, USA) (extinction coefficient for NB7, $31,650 \text{ M}^{-1} \text{ cm}^{-1}$; for NB8 and NB37, $31,525 \text{ M}^{-1} \text{ cm}^{-1}$).

5.2. PSMA monomer-dimer equilibrium

To validate the conformation of PSMA (residues 44–750; purchased from Caltech Protein Expression Center), SEC analysis using a Superdex 200 16/600 column (GE Healthcare, MA, USA) was performed. The protocol for purifying the dimeric and monomeric forms of PSMA was adapted from Schülke et al. [8]. In brief, a mixture of the monomer and dimer forms of PSMA underwent buffer exchange into PBS containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+} (designated PBS+) supplemented with 2 M NaCl, and then incubated for 5 days at 4 °C, resulting in a conformational change to the dimer (*SI Appendix*, Fig. S2). The mixed sample containing both the dimeric and monomeric conformations (as seen in *SI Appendix*, Fig. S2) was subjected to buffer exchange to PBS lacking calcium and magnesium supplements but supplemented with 2 mM of EDTA, and then incubated for 5 days at 4 °C. This buffer exchange converted the dimer into a monomer, indicating that the stability of the dimer depends on the presence of metal ions, such as Zn^{2+} , in the active site of PSMA. To validate the conformational shift, a second SEC analysis was performed. Monomeric and dimeric PSMA fractions were collected, concentrated, and stored at -80°C .

5.3. Preparation of NB-PSMA complexes

Each NB was incubated at room temperature for 1 h with PSMA dimer at the following molar ratios: 1:10 (PSMA:NB7), 1:6 (PSMA:NB37), 1:3 (PSMA:NB8) or 1:5.6:5.6 (PSMA:NB7:NB8). To separate the complexes from free NBs, an HiLoad 16/600 Superdex 200 pg (GE Healthcare) column was used. The size and purity of the complexes were evaluated using 12.5 % SDS-PAGE and 12 % native-PAGE gel

electrophoresis.

5.4. Cryo-EM data collection, structure determination and refinement

Mixtures of NBs and PSMA, 3 μL each, at a concentration of ~ 1 mg/mL of the mixture, were deposited on glow-discharged Quantifoil R 1.2/1.3 holey carbon grids (Quantifoil Micro Tools GmbH, Germany). Samples were manually blotted for 3 s at room temperature following vitrification by plunging them rapidly into liquid ethane using an in-house-built plunging apparatus. The frozen samples were stored in liquid nitrogen until imaging. Samples were loaded under cryogenic conditions and imaged in low-dose mode on a Glacios microscope (Thermo-Fisher) operated at 200 kV. Automated data collection was performed using EPU (Thermo-Fisher). Movies were collected at a calibrated pixel size of 0.89 Å using a Falcon 4i direct electron detector fitted behind a Selectris X energy filter (Thermo-Fisher) set to ± 5 eV around a zero-loss peak. The detector was operated in a dose fractionated counting mode, at a dose rate of 8 $\text{e}^-/\text{pixel}/\text{s}$, with a total electron dose of 30 $\text{e}^-/\text{Å}^2$. Data were collected at a defocus range of -0.5 to -2.0 μm . Processing was done using cryoSPARC v4.3.0 [53]. Dose-fractionated movie stacks were patch-motion corrected, and their defocus values were determined. The aligned sum images were used for further processing. Particles were picked with a template picker, followed by extraction at a box size of 256 pixels. 2D class averages were manually selected and used for further 3D refinement. Ab-initio reconstruction was used to prepare an initial map for further refinement. Models were built into the 3D map using Coot 0.9.8 [54] and refined in Coot and Phenix1.20-4444 [55]. The obtained cryo-EM maps and atomic models were visualized using open-source UCSF Chimera and ChimeraX [56,57]. The validation statistics of the obtained atomic models are shown in *SI Appendix*, Table S1.

5.5. Surface plasmon resonance (SPR) experiment for dual-binding evaluation

The interaction between PSMA and NB7 or NB8 was characterized using a ProteOn™ XPR36 instrument (Bio-Rad Laboratories, CA, USA). NB7 was immobilized on a GLC chip (Bio-Rad Laboratories) using standard amino coupling procedures, including activation of the carboxymethyl groups on the surface of the sensor chip with 20 mM EDAC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride] and 5 mM S-NHS (*N*-hydroxysuccinimide), and run at 30 $\mu\text{L}/\text{min}$ for 300 s. Thereafter, 9 μg of NB7 were suspended in acetate buffer, pH 4.5 [the low pH enhances the efficiency of immobilization during the amine coupling procedure [58]] and run at 30 $\mu\text{L}/\text{s}$ for 300 s to ensure optimal occupancy of the active sites on the sensor surface. Bovine serum albumin (BSA) (3 μg) suspended in acetate buffer, pH 4.5, was run on another channel as a negative control. Empty carboxyl residues were deactivated with 1 M ethanolamine at 30 $\mu\text{L}/\text{min}$ for 300 s. All reactions were performed at 25 °C in PBST (PBS x1 + 0.005 % Tween 20) as the running buffer. PSMA (66 nM) was injected as an analyte into channels 1–4 at 30 $\mu\text{L}/\text{min}$ for 400 s, while PBST was injected into channels 5 and 6 as a control to verify the absence of direct interaction between the two NBs. Thereafter, NB8 (112 nM) was injected at 30 $\mu\text{L}/\text{min}$ for 332 s into channels 1, 2 and 5. In parallel, PBST buffer was run on the other channels (3,4 and 6) as a background control. Data were analyzed on the ProteOn manager software version 3.01.

5.6. SPR binding assay for k_{on} , k_{off} and K_D determination

SPR measurements were performed on a Biacore T200 instrument. NB7 (20 $\mu\text{g}/\text{mL}$) was immobilized on a CM5 chip using an amine coupling kit (GE Healthcare, Chicago, IL, USA). The analytes, recombinant human PSMA/FOLH1 Protein, His Tag (Sino Biological, Beijing, China) or murine PSMA/FOLH1 Protein, His Tag (ACROBiosystems, Newark, Delaware, USA) dissolved in PBST (PBS supplemented with

0.005 % Tween-20 v/v), were allowed to flow over the chip at 30 $\mu\text{L}/\text{min}$ in various concentrations (0–64 nM), and the chip was regenerated using 10 mM glycine-HCl at pH 2.0 (Cytiva, Marlborough, MA, USA). Binding was allowed for 120 s, and dissociation, for 600 s. Curves were fitted by applying Biacore's evaluation software using a 1:1 ligand:analyte binding model. From this, the association rate constant (k_{on}), dissociation rate constant (k_{off}), and equilibrium dissociation constant (K_D) were derived.

5.7. Cell binding assay

PC3-PIP (PSMA⁺) cells were grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS), 1 % L-glutamine and 1 % penicillin-streptomycin (Biological Industries, Israel). Once the cells reached ~ 80 % confluence, samples of 50,000 cells were transferred into each of a number of Eppendorf tubes, as required by the number of samples and controls in the experiment. Fluorescently labeled NBs (NB7-AF647 and NB8-DyLight™ 488) were added to the cells individually or in combination at concentrations of 0.1, 0.5, 2, 5, 10, 20, 50, 100, 500, or 1000 nM. The cells were incubated with the NBs for 1 h at 4 °C on tube rotators, followed by three PBS-FBS (2.5 %) washing steps (350 g for 5 min). The cells were kept on ice throughout the experiment. The experiment was performed in triplicate. The fluorescence of each sample was measured using an Accuri C6 flow cytometry analyzer (BD Biosciences, CA, USA).

CRediT authorship contribution statement

Gal Alon-Zchut: Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Ran Zalk:** Writing – review & editing, Validation, Software, Formal analysis, Data curation. **Truc T. Huynh:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Michael R. Zalutsky:** Writing – review & editing, Supervision, Formal analysis. **Yossi Weizmann:** Writing – review & editing, Supervision, Conceptualization. **Raz Zarivach:** Writing – review & editing, Validation, Software, Formal analysis. **Niv Papo:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Niv Papo reports financial support was provided by Israel Cancer Research Fund. Niv Papo reports financial support was provided by United States-Israel Binational Science Foundation. Niv Papo reports financial support was provided by National Institutes of Health. Niv Papo reports financial support was provided by Rosetrees. Niv Papo reports financial support was provided by Israel Science Foundation. Niv Papo reports financial support was provided by Worldwide Cancer Research. Niv Papo has patent A SINGLE-DOMAIN ANTIBODY FOR TARGETING PROSTATE SPECIFIC MEMBRANE ANTIGEN (PSMA) issued to NIBN. Niv Papo has patent CONJUGATES AND USE OF SAME FOR THERANOSTICS pending to NIBN. Michael Zalutsky has patent CONJUGATES AND USE OF SAME FOR THERANOSTICS pending to Duke University. None if there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2025.145693>.

References

- [1] J. Ferlay, M. Ervik, F. Lam, M. Laversanne, M. Colombet, L. Mery, M. Piñeros, A. Znaor, I. Soerjomataram, F. Bray, Global cancer observatory: Cancer today. <https://Gco.larc.Who.Int/Today> 2024(Apr 21), 2024.
- [2] Y.H.W. Derks, et al., PSMA-targeting agents for radio- and fluorescence-guided prostate cancer surgery, *Theranostics* 9 (23) (2019) 6824–6839.
- [3] A.W. Hensbergen, et al., Image-guided surgery: are we getting the most out of small-molecule prostate-specific-membrane-antigen-targeted tracers? *Bioconjug. Chem.* 31 (2) (2019) 375–395.
- [4] W. Jones, K. Griffiths, P.C. Barata, C.J. Paller, PSMA theranostics: review of the current status of PSMA-targeted imaging and radioligand therapy, *Cancers* 12 (6) (2020).
- [5] F. Bois, et al., [68Ga]ga-PSMA-11 in prostate cancer: a comprehensive review, *Am. J. Nucl. Med. Mol. Imaging* 10 (6) (2020) 349–374.
- [6] M.A. Puentes, L.A. Mandujano, L.R. Guarenos, E. Azorín, D. Osorio-González, In silico analysis of the structural properties of PSMA and its energetic relationship with Zn as cofactor, *Journal of Nuclear Physics, Material Science, Radiation and Application* 6 (2018) 115–120.
- [7] M.I. Davis, M.J. Bennett, L.M. Thomas, P.J. Bjorkman, Crystal structure of prostate-specific membrane antigen, a tumor marker and peptidase, *Proc. Natl. Acad. Sci.* 102 (17) (2005) 5981–5986.
- [8] N. Schülke, et al., The homodimer of prostate-specific membrane antigen is a functional target for cancer therapy, *Proc. Natl. Acad. Sci. USA* 100 (22) (2003) 12590–12595.
- [9] V. Yao, D.J. Bacich, Prostate specific membrane antigen (PSMA) expression gives prostate cancer cells a growth advantage in a physiologically relevant folate environment in vitro, *Prostate* 66 (8) (2006) 867–875.
- [10] V. Yao, C.E. Berkman, J.K. Choi, D.S. O'Keefe, D.J. Bacich, Expression of prostate-specific membrane antigen (PSMA), increases cell folate uptake and proliferation and suggests a novel role for PSMA in the uptake of the non-polyglutamated folate, folic acid, *Prostate* 70 (3) (2010) 305–316.
- [11] R.G. Lapidus, C.W. Tiffany, J.T. Isaacs, B.S. Slusher, Prostate-specific membrane antigen (PSMA) enzyme activity is elevated in prostate cancer cells, *Prostate* 45 (4) (2000) 350–354.
- [12] H. Liu, et al., Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor vascular Endothelium1, 1997.
- [13] N.H. Bander, et al., Phase I trial of 177Lutetium-labeled J591, a monoclonal antibody to prostate-specific membrane antigen, in patients with androgen-independent prostate cancer, *JCO* 23 (21) (2005) 4591.
- [14] A. Ghosh, W.D.W. Heston, Tumor target prostate specific membrane antigen (PSMA) and its regulation in prostate cancer, *J. Cell. Biochem.* 91 (3) (2003) 528.
- [15] S. Moffett, et al., Preparation and characterization of new anti-PSMA monoclonal antibodies with potential clinical use, *Hybridoma* 26 (6) (2007) 363.
- [16] M.J. Morris, et al., Phase I evaluation of J591 as a vascular targeting agent in progressive solid tumors, *Clin. Cancer Res.* 13 (9) (2007) 2707.
- [17] N. Pandit-Taskar, et al., Indium 111-labeled J591 anti-PSMA antibody for vascular targeted imaging in progressive solid tumors, *EJNMMI Res.* 5 (1) (2015).
- [18] S. Vallabhajosula, et al., Radioimmunotherapy of prostate cancer using 90Y- and 177Lu-labeled J591 monoclonal antibodies: effect of multiple treatments on myelotoxicity, *Clin. Cancer Res.* 11 (19) (2004) 7195s.
- [19] S.T. Tagawa, et al., Phase 1/2 study of fractionated dose lutetium-177-labeled anti-prostate-specific membrane antigen monoclonal antibody J591 (177Lu-J591) for metastatic castration-resistant prostate cancer, *Cancer* 125 (15) (2019) 2561.
- [20] J.S. Batra, et al., Phase I trial of docetaxel plus lutetium-177-labeled anti-prostate-specific membrane antigen monoclonal antibody J591 (177Lu-J591) for metastatic castration-resistant prostate cancer, *Urologic Oncology: Seminars and Original Investigations* 38 (11) (2020) 848.
- [21] M.J. Niaz, et al., Pilot study of hyperfractionated dosing of lutetium-177-labeled anti-prostate-specific membrane antigen monoclonal antibody J591 (177Lu-J591) for metastatic castration-resistant prostate cancer, *Oncologist* 25 (6) (2024) 477.
- [22] N.M. Donin, R.E. Reiter, Why targeting PSMA is a game changer in the management of prostate cancer, *J. Nucl. Med.* 59 (2) (2018) 177–182.
- [23] D. Xiang, et al., Superior performance of aptamer in tumor penetration over antibody: implication of aptamer-based theranostics in solid tumors, *Theranostics* 5 (10) (2015) 1083–1097.
- [24] Ilse Vaneycken, et al., Immuno-imaging using nanobodies - ScienceDirect, *Curr. Opin. Biotechnol.* 22 (6) (2011) 877–881.
- [25] I. Jovčević, S. Muyldermans, The therapeutic potential of nanobodies, *BioDrugs* 34 (1) (2019) 11.
- [26] L. Rosenfeld, et al., Nanobodies targeting prostate-specific membrane antigen for the imaging and therapy of prostate cancer, *J. Med. Chem.* 63 (14) (2020) 7601–7615.
- [27] T.T. Huynh, et al., PSMA-reactive NB7 single domain antibody fragment: a potential scaffold for developing prostate cancer theranostics, *Nucl. Med. Biol.* 134–135 (2024) 108913.
- [28] J. Govaert, et al., Dual beneficial effect of interloop disulfide bond for single domain antibody fragments *, *J. Biol. Chem.* 287 (3) (2012) 1970–1979.
- [29] A. Manglik, B.K. Kobilka, Steyaert, J2 Nanobodies to study G Protein-Coupled receptor structure and function. *Annual Review of Pharmacology and Toxicology*, 57, Volume 57, 2017, pp. 19–37.
- [30] P. Kunz, et al., The structural basis of nanobody unfolding reversibility and thermoresistance, *Sci. Rep.* 8 (1) (2018).
- [31] U. Hennrich, M. Eder, [177Lu] lu-PSMA-617 (PluvictoTM): the first FDA-approved radiotherapeutic for treatment of prostate cancer, *Pharmaceuticals* 15 (10) (2022) 1292.
- [32] T. Chou, Drug combination studies and their synergy quantification using the chou-talalay method, *Cancer Res.* 70 (2) (2010) 440–446.
- [33] C. Holohan, S. Van Schaeybroeck, D.B. Longley, P.G. Johnston, Cancer drug resistance: an evolving paradigm, *Nat. Rev. Cancer* 13 (10) (2013) 714–726.
- [34] J. Jumper, et al., Highly accurate protein structure prediction with AlphaFold, *Nature* 596 (7873) (2021) 583–589.
- [35] K. Hlouchova, C. Barinka, J. Konvalinka, J. Lubkowski, Structural insight into the evolutionary and pharmacologic homology of glutamate carboxypeptidase II and III, *FEBS J.* 276 (16) (2009) 4448–4462.
- [36] J. Tykvart, et al., Rational design of urea-based glutamate carboxypeptidase II (GCPII) inhibitors as versatile tools for specific drug targeting and delivery, *Bioorg. Med. Chem.* 22 (15) (2014) 4099–4108.
- [37] C. Barinka, et al., Interactions between human glutamate carboxypeptidase II and urea-based inhibitors: structural characterization, *J. Med. Chem.* 51 (24) (2008) 7737–7743.
- [38] J. Zhang, et al., A prostate-specific membrane antigen activated molecular rotor for real-time fluorescence imaging, *Nat. Commun.* 12 (1) (2021) 5460–5466.
- [39] J. Ptacek, et al., Structural basis of prostate-specific membrane antigen recognition by the A9g RNA aptamer, *Nucleic Acids Res.* 48 (19) (2020) 11130–11145.
- [40] X. Bai, G. McMullan, S.H.W. Scheres, How cryo-EM is revolutionizing structural biology, *Trends Biochem. Sci.* 40 (1) (2015) 49–57.
- [41] J.S. Huston, et al., Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*, *Proc. Natl. Acad. Sci.* 85 (16) (1988) 5879–5883.
- [42] H. Gursahani, J. Riggs-Sauthier, J. Pfeiffer, D. Lechuga-Ballesteros, C.S. Fishburn, Absorption of polyethylene glycol (PEG) polymers: the effect of PEG size on permeability, *J. Pharm. Sci.* 98 (8) (2009) 2847–2856.
- [43] A. Erijman, E. Rosenthal, J.M. Shifman, How structure defines affinity in protein-protein interactions, *PLoS One* 9 (10) (2014) e110085.
- [44] A. Cooper, D.T.F. Dryden, Allostery without conformational change - a plausible model, *Eur. Biophys. J.* 11 (2) (1984) 103–109.
- [45] E.A. Ruigrok, et al., Extensive preclinical evaluation of lutetium-177-labeled PSMA-specific tracers for prostate cancer radionuclide therapy, *Eur. J. Nucl. Med. Mol. Imaging* 48 (2021) 1339–1350.
- [46] M.J. Uijen, et al., Kidney absorbed radiation doses for [177Lu] lu-PSMA-617 and [177Lu] lu-PSMA-I&T determined by 3D clinical dosimetry, *Nucl. Med. Commun.* 44 (4) (2023) 270–275.
- [47] H.E. White, A. Ignatiou, Clare DK & Orlova EV (2017) structural study of heterogeneous biological samples by cryoelectron microscopy and image processing, *Biomed. Res. Int.* 1 (2017) 1032432.
- [48] J.N. Rabuck-Gibbons, D. Lyumkis, J.R. Williamson, Quantitative mining of compositional heterogeneity in cryo-EM datasets of ribosome assembly intermediates, *Structure* 30 (4) (2022), 498–509. e4.
- [49] D. Kimanius, J. Schwab, Confronting heterogeneity in cryogenic electron microscopy data: innovative strategies and future perspectives with data-driven methods, *Curr. Opin. Struct. Biol.* 86 (2024) 102815.
- [50] C. Ackaert, et al., Immunogenicity risk profile of nanobodies, *Front. Immunol.* 12 (2021) 632687.
- [51] M.A. Rossotti, K. Bélanger, K.A. Henry, J. Tanha, Immunogenicity and humanization of single-domain antibodies, *FEBS J.* 289 (14) (2022) 4304–4327.
- [52] P.H. Kuo, et al., Quantitative 68Ga-PSMA-11 PET and clinical outcomes in metastatic castration-resistant prostate cancer following 177Lu-PSMA-617 (VISION trial), *Radiology* 312 (2) (2024) e233460.
- [53] A. Punjani, J.L. Rubinstein, D.J. Fleet, M.A. Brubaker, cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination, *Nat. Methods* 14 (3) (2017) 290–296.
- [54] P. Emsley, B. Lohkamp, W. Scott, K. Cowtan, Features and development of coot, *Acta Crystallogr. Sect. D* 66 (2010) 486–501.
- [55] D. Liebschner, et al., Macromolecular structure determination using X-rays, neutrons and electrons: recent developments in phenix, *Acta Crystallographica Section D: Structural Biology* 75 (10) (2019) 861–877.
- [56] E.F. Pettersen, et al., UCSF chimera—a visualization system for exploratory research and analysis, *J. Comput. Chem.* 25 (13) (2004) 1605–1612.

- [57] E.F. Pettersen, et al., UCSF ChimeraX: structure visualization for researchers, educators, and developers, *Protein Sci.* 30 (1) (2021) 70–82.
- [58] J. Ezzati Nazhad Dolatabadi, M. de la Guardia, Tips on ligand immobilization and kinetic study using surface plasmon resonance, *Bioimpacts* 6 (3) (2016) 117–118.
- [59] J. Chen, N. Sawyer, L. Regan, Protein–protein interactions: general trends in the relationship between binding affinity and interfacial buried surface area, *Protein Sci.* 22 (4) (2013) 510.