International Vaccine Design Center

Division of Vaccine Engineering (New Dimensional Vaccine Design Team)

新次元ワクチンデザイン系・ワクチン工学分野

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Biomolecular recognition is based on collective and specific non-covalent interactions between discrete biological molecules. Our laboratory studies various protein systems, for instance, antibody-antigen and protein-ligand complexes, to understand quantitatively how these coordinated non-covalent interactions contribute to their specific recognition in biological and artificial systems. We seek to elucidate the molecular mechanisms by which biological molecules obtain high-specificity and affinity from multiple angles using advanced instrumentation. To produce functional molecules with higher performance and better properties, we aim to build a solid foundation from which to develop drugs that modulate specific interactions between biomolecules and ultimately to understand the principles of molecular interactions in our lives.

1. Development of a high-throughput method to screen novel antiviral materials

Nakakido M, Tanaka N, Shimojo A, Miyamae N, Tsumoto K.

Respiratory infectious diseases pose a serious threat worldwide, and novel antiviral materials are highly demanded. Photocatalytic nanoparticles have been developed to inhibit indirect transmission of pathogens by acting as surface coating materials. During development of such antiviral materials, researchers use bacteriophages as model viruses due to their safety and experimental efficiency. Screening methods are used to identify potential antiviral materials, and better screening technologies will accelerate the discovery of antiviral treatments. In this study, we constructed a novel platform to evaluate antiviral activity of surface coating materials using the M13 bacteriophage and phagemid system derived from phage display technology. The evaluation results generated by this system for the two tested antiviral materials were comparable to those for the materials tested on the Qβ bacteriophage and influenza virus using traditional screening methods. The experimental system developed in this study provides rapid and effective screening and can be applied to the development of novel antiviral materials.

2. Oligo (N-methylalanine) as a Peptide-Based Molecular Scaffold with a Minimal Structure and High Density of Functionalizable Sites

Yokomine M, Morimoto J, Fukuda Y, Shiratori Y, Kuroda D, Ueda T, Takeuchi K, Tsumoto K, Sando S.

Functionalizable synthetic molecules with nanometer sizes and defined shapes in water are useful as molecular scaffolds to mimic the functions of biomacromolecules and develop chemical tools for manipulating biomacromolecules. Herein, we propose oligo (N-methylalanine) (oligo-NMA) as a peptide-based molecular scaffold with a minimal structure and a

high density of functionalizable sites. Oligo-NMA forms a defined shape in water without hydrogen-bonding networks or ring constraints, which enables the molecule to act as a scaffold with minimal atomic composition. Furthermore, functional groups can be readily introduced on the nitrogens and α-carbons of oligo-NMA. Computational and NMR spectroscopic analysis suggested that the backbone structure of oligo-NMA is not largely affected by functionalization. Moreover, the usefulness of oligo-NMA was demonstrated by the design of protein ligands. The ease of synthesis, minimal structure, and high functionalization flexibility makes oligo-NMA a useful scaffold for chemical and biological applications.

3. Biophysical Characterization of the Contribution of the Fab Region to the IgG-FcγRIIIa Interaction

Kosuge H, Nagatoishi S, Kiyoshi M, Ishii-Watabe A, Terao Y, Ide T, Tsumoto K.

The cell-surface receptor FcγRIIIa is crucial to the efficacy of therapeutic antibodies as well as the immune response. The interaction of the Fc region of IgG molecules with FcγRIIIa has been characterized, but until recently, it was thought that the Fab regions were not involved in the interaction. To evaluate the influence of the Fab regions in a biophysical context, we carried out surface plasmon resonance analyses using recombinant FcγRIIIa ligands. A van't Hoff analysis revealed that compared to the interaction of the papain-digested Fc fragment with FcγRIIIa, the interaction of commercially available, full-length rituximab with FcγRIIIa had a more favorable binding enthalpy, a less favorable binding entropy, and a slower off rate. Similar results were obtained from analyses of IgG1 molecules and an IgG1-Fc fragment produced by Expi293 cells. For further validation, we also prepared a maltose-binding protein-linked IgG1-Fc fragment (MBP-Fc). The binding enthalpy of MBP-Fc was nearly equal to that of the IgG1-Fc fragment for the interaction with FcγRIIIa, indicating that such alternatives to the Fab domains as MBP do not positively contribute to the IgG-FcγRIIIa interactions. Our investigation strongly suggests that the Fab region directly interacts with FcγRIIIa, resulting in an increase in the binding enthalpy and a decrease in the dissociation rate, at the expense of favorable binding entropy.

4. Mirror-image streptavidin with specific binding to L-biotin, the unnatural enantiomer

Suganuma M, Kubo T, Ishiki K, Tanaka K, Suto K, Ejima D, Toyota M, Tsumoto K, Sato T, Nishikawa Y.

The streptavidin-biotin system is known to have a very high affinity and specificity and is widely used in biochemical immunoassays and diagnostics. However, this method is affected by endogenous D-biotin in serum sample measurements (biotin interference). While several efforts using alternative high-affinity binding systems (e.g., genetically modified streptavidin and biotin derivatives) have been attempted, these efforts have all led to reduction in affinity. To solve this interference issue, the enantiomer of streptavidin was synthesized, which enabled specific binding to L-biotin. We successfully obtained a functional streptavidin molecule by peptide synthesis using D-amino acids and an in vitro folding technique. Several characterizations, including size exclusion chromatography (SEC), circular dichroism spectra (CD), and heat denaturation experiments collectively confirmed the higher-order enantiomer of natural streptavidin had been formed with comparable stability to the natural protein. L-biotin specific binding of this novel molecule enabled us to avoid biotin interference in affinity measurements using the Biacore system and enzyme-linked immunosorbent assay (ELISA). We propose the enantiomer of streptavidin as a potential candidate to replace the natural streptavidin-biotin system, even for in vivo use.

5. Experimental Comparison of Bond Lifetime and Viscoelastic Relaxation in Transient Networks with Well-Controlled Structures

Katashima T, Kudo R, Naito M, Nagatoishi S, Miyata K, Chung UI, Tsumoto K, Sakai T.

We demonstrate an experimental comparison of the bond lifetime, estimated using surface plasmon resonance (SPR), and the viscoelastic relaxation time of transient networks with well-controlled structures (dynamically cross-linked Tetra-PEG gel). SPR and viscoelastic measurements revealed that the temperature dependences of the two characteristic times are in agreement, while the viscoelastic response is delayed with respect to the lifetime by a factor of 2-3, dependent on the network strand length. Polymers cross-linked by temporary interactions form transient networks, which show fascinating viscoelasticity with a single relaxation mode. However, the molecular understanding of such simple viscoelasticity has remained incomplete because of the difficulty of experimentally evaluating bond lifetimes and heterogeneous structures in conventional transient networks. Our results suggest that bond dissociation and recombination both contribute to the macromechanical response. This report on direct bond-lifetime-viscoelastic-relaxation time comparison provides important information for the molecular design of transient network materials.

6. Antibody recognition of complement factor H reveals a flexible loop involved in atypical hemolytic uremic syndrome pathogenesis

Yokoo T, Tanabe A, Yoshida Y, Caaveiro JMM, Nakakido M, Ikeda Y, Fujimura Y, Matsumoto M, Entzminger K, Maruyama T, Okumura CJ, Nangaku M, Tsumoto K.

Atypical hemolytic uremic syndrome (aHUS) is a disease associated with dysregulation of the immune complement system, especially of the alternative pathway (AP). Complement factor H (CFH), consisting of 20 domains called complement control protein (CCP1-20), downregulates the AP as a cofactor for mediating C3 inactivation by complement factor I. However, anomalies related to CFH are known to cause excessive complement activation and cytotoxicity. In aHUS, mutations and the presence of anti-CFH autoantibodies (AAbs) have been reported as plausible causes of CFH dysfunction, and it is known that CFH-related aHUS carries a high probability of end-stage renal disease. Elucidating the detailed functions of CFH at the molecular level will help to understand aHUS pathogenesis. Herein, we used biophysical data to reveal that a heavy-chain antibody fragment, termed VHH4, recognized CFH with high affinity. Hemolytic assays also indicated that VHH4 disrupted the protective function of CFH on sheep erythrocytes. Furthermore, X-ray crystallography revealed that VHH4 recognized the Leu1181-Leu1189CCP20 loop, a known anti-CFH AAbs epitope. We next analyzed the dynamics of the C-terminal region of CFH and showed that the epitopes recognized by anti-CFH AAbs and VHH4 were the most flexible regions in CCP18-20. Finally, we conducted mutation analyses to elucidate the mechanism of VHH4 recognition of CFH and revealed that VHH4 inserts the Trp1183C-CP20 residue of CFH into the pocket formed by the complementary determining region 3 loop. These results suggested that anti-CFH AAbs may adopt a similar molecular mechanism to recognize the flexible loop of Leu1181-Leu1189CCP20, leading to aHUS pathogenesis.

7. Structure and role of the linker domain of the iron surface-determinant protein IsdH in heme transportation in Staphylococcus aureus

Valenciano-Bellido S, Caaveiro JMM, Morante K, Sushko T, Nakakido M, Nagatoishi S, Tsumoto K.

Staphylococcus aureus is a major cause of deadly nosocomial infections, a severe problem fueled by the steady increase of resistant bacteria. The iron surface determinant (Isd) system is a family of proteins that acquire nutritional iron from the host organism, helping the bacterium to proliferate during infection, and therefore represents a promising antibacterial target. In particular, the surface protein IsdH captures hemoglobin (Hb) and acquires the heme moiety containing the iron atom. Structurally, IsdH comprises three distinctive NEAr-iron Transporter (NEAT) domains connected by linker domains. The objective of this study was to characterize the linker region between NEAT2 and NEAT3 from various biophysical viewpoints and thereby advance our understanding of its role in the molecular mechanism of heme extraction. We demonstrate the linker region contributes to the stability of the bound protein, likely influencing the flexibility and orientation of the NEAT3 domain in its interaction with Hb, but only exerts a modest contribution to the affinity of IsdH for heme. Based on these data, we suggest that the flexible nature of the linker facilitates the precise positioning of NEAT3 to acquire heme. In addition, we also found that residues His45 and His89 of Hb located in the heme transfer route toward IsdH do not play a critical role in the transfer rate-determining step. In conclusion, this study clarifies key elements of the mechanism of heme extraction of human Hb by IsdH, providing key insights into the Isd system and other protein systems containing NEAT domains.

8. Addition of arginine hydrochloride and proline to the culture medium enhances recombinant protein expression in Brevibacillus choshinensis: The case of RBD of SARS-CoV-2 spike protein and its antibody

Matsunaga R, Tsumoto K.

Brevibacillus choshinensis is a gram-positive bacterium that is known to efficiently secrete recombinant proteins. However, the expression of these proteins is often difficult depending upon the expressed protein. In this study, we demonstrated that the addition of arginine hydrochloride and proline to the culture medium dramatically increased protein expression. By culturing bacterial cells in 96-well plates, we were able to rapidly examine the expression conditions and easily scale up to 96 mL of culture for production. Although functional expression of the receptor binding domain (RBD) of the SARS-CoV-2 spike protein without any solubility-enhancing tag in bacterial strains (including Escherichia coli) has not been reported to date, we succeeded in efficiently producing RBD which showed a similar CD spectrum to that of RBD produced by eukaryotic cell expression systems. Furthermore, RBD from the omicron variant (B.1.1.529) was also produced. Physicochemical analyses indicated that omicron RBD exhibited markedly increased instability compared to the wild-type. We also revealed that the Fab format of the anti-SARS-CoV-2 antibody C121 can be produced in large quantities using the same expression system. The obtained C121 Fab bound to wild-type RBD but not to omicron RBD. These results strongly suggest that the Breviba-

cillus expression system is useful for facilitating the efficient expression of proteins that are difficult to fold and will thus contribute to the rapid physicochemical evaluation of functional proteins.

9. Ladder observation of bovine serum albumin by high resolution agarose native gel electrophoresis

Tomioka Y, Nakagawa M, Sakuma C, Nagatoishi S, Tsumoto K, Arakawa T, Akuta T.

A commercially available bovine serum albumin (BSA) was examined by agarose native gel electrophoresis using two different agarose sources, UltraPure and MetaPhor agarose. While UltraPure agarose up to 5 % showed no clear separation of BSA oligomers, MetaPhor agarose clearly demonstrated oligomer bands above 4 %, indicating that the latter agarose has greater molecular sieving effects and is hence characterized to have high resolution for size differences, as probed by a greater slope of Ferguson plot. Physical properties are different between two agaroses. In general, UltraPure agarose has physical strength, while MetaPhor agarose is considerably fragile, but MetaPhor agarose solution is less viscous so that even 10 % gel can be made. Cause of oligomers was shown to be not associated with inter-chain disulfide bonds, but is due to association of native or native-like molecules.

10. Residue-based program of a β-peptoid twisted strand shape via a cyclopentane constraint

Kim J, Kobayashi H, Yokomine M, Shiratori Y, Ueda T, Takeuchi K, Umezawa K, Kuroda D, Tsumoto K, Morimoto J, Sando S.

N-Substituted peptides, such as peptoids and β-peptoids, have been reported to have unique structures with diverse functions, like catalysis and manipulation of biomolecular functions. Recently, the preorganization of monomer shape by restricting bond rotations about all backbone dihedral angles has been demonstrated to be useful for de novo design of peptoid structures. Such design strategies are hitherto unexplored for β-peptoids; to date, no preorganized β-peptoid monomers have been reported. Here, we report the first design strategy for β-peptoids, in which all four backbone dihedral angles (ω, ϕ, θ, ψ) are rotationally restricted on a per-residue basis. The introduction of a cyclopentane constraint realized the preorganized monomer structure and led to a β-peptoid with a stable twisted strand shape.

11. PRELP Regulates Cell-Cell Adhesion and EMT and Inhibits Retinoblastoma Progression

Hopkins J, Asada K, Leung A, Papadaki V, Davaapil H, Morrison M, Orita T, Sekido R, Kosuge H, Reddy MA, Kimura K, Mitani A, Tsumoto K, Hamamoto R, Sagoo MS, Ohnuma SI.

Retinoblastoma (RB) is the most common intraocular pediatric cancer. Nearly all cases of RB are associated with mutations compromising the function of the RB1 tumor suppressor gene. We previously demonstrated that PRELP is widely downregulated in various cancers and our in vivo and in vitro analysis revealed PRELP as a novel tumor suppressor and regulator of EMT. In addition, PRELP is located at chromosome 1q31.1, around a region hypothesized to be associated with the initiation of malignancy in RB. Therefore, in this study, we investigated the role of PRELP in RB through in vitro analysis and next-generation sequencing. Immunostaining revealed that PRELP is expressed in Müller glial cells in the retina. mRNA expression profiling of PRELP-/- mouse retina and PRELP-treated RB cells found that PRELP contributes to RB progression via regulation of the cancer microenvironment, in which loss of PRELP reduces cell-cell adhesion and facilitates EMT. Our observations suggest that PRELP may have potential as a new strategy for RB treatment.

12. Analysis of bovine serum albumin unfolding in the absence and presence of ATP by SYPRO Orange staining of agarose native gel electrophoresis

Tomioka Y, Nakagawa M, Sakuma C, Kurosawa Y, Nagatoishi S, Tsumoto K, Arakawa T, Akuta T.

An attempt was made to specifically stain unfolded proteins on agarose native gels. SYPRO Orange is routinely used to detect unfolded protein in differential scanning fluorimetry, which is based on the enhanced fluorescence intensity upon binding to the unfolded protein. We demonstrated that this dye barely bound to the native proteins, resulting in no or faint staining of the native bands, but bound to and stained the unfolded proteins, on agarose native gels. Using bovine serum albumin (BSA), it was shown that staining did not depend on whether BSA was thermally unfolded in the presence of SYPRO Orange or stained after electrophoresis. On the contrary, SYPRO Orange dye stained protein bands in the presence of sodium dodecylsulfate (SDS) due to incorporation of the dye into SDS micelles that bound to the unfolded proteins. This staining resulted in detection of new, intermediately unfolded structure of BSA during thermal unfolding. Such intermediate structure occurred at higher temperature in the presence of ATP.

13. Human antibody recognition and neutralization mode on the NTD and RBD domains of SARS-CoV-2 spike protein

Otsubo R, Minamitani T, Kobiyama K, Fujita J, Ito T, Ueno S, Anzai I, Tanino H, Aoyama H, Matsuura Y, Namba K, Imadome KI, Ishii KJ, Tsumoto K, Kamitani W, Yasui T.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes coronavirus disease 2019 (COVID-19). Variants of concern (VOCs) such as Delta and Omicron have developed, which continue to spread the pandemic. It has been reported that these VOCs reduce vaccine efficacy and evade many neutralizing monoclonal antibodies (mAbs) that target the receptor binding domain (RBD) of the glycosylated spike (S) protein, which consists of the S1 and S2 subunits. Therefore, identification of optimal target regions is required to obtain neutralizing antibodies that can counter VOCs. Such regions have not been identified to date. We obtained 2 mAbs, NIBIC-71 and 7G7, using peripheral blood mononuclear cells derived from volunteers who recovered from COVID-19. Both mAbs had neutralizing activity against wild-type SARS-CoV-2 and Delta, but not Omicron. NIBIC-71 binds to the RBD, whereas 7G7 recognizes the N-terminal domain of the S1. In particular, 7G7 inhibited S1/S2 cleavage but not the interaction between the S protein and angiotensin-converting enzyme 2; it suppressed viral entry. Thus, the efficacy of a neutralizing mAb targeting inhibition of S1/2 cleavage was demonstrated. These results suggest that neutralizing mAbs targeting blockade of S1/S2 cleavage are likely to be cross-reactive against various VOCs.

14. Molecular basis for thermal stability and affinity in a VHH: Contribution of the framework region and its influence in the conformation of the CDR3

Kinoshita S, Nakakido M, Mori C, Kuroda D, Caaveiro JMM, Tsumoto K.

The camelid single domain antibody, referred to VHH or Nanobody, is considered a versatile tool for various biotechnological and clinical applications because of its favorable biophysical properties. To take advantage of these characteristics and for its application in biotechnology and therapy, research on VHH engineering is currently vigorously conducted. To humanize a camelid VHH, we performed complementarity determining region (CDR) grafting using a humanized VHH currently in clinical trials, and investigated the effects of these changes on the biophysical properties of the resulting VHH. The chimeric VHH exhibited a significant decrease in affinity and thermal stability and a large conformational change in the CDR3. To elucidate the molecular basis for these changes, we performed mutational analyses on the framework regions revealing the contribution of individual residues within the framework region. It is demonstrated that the mutations resulted in the loss of affinity and lower thermal stability, revealing the significance of bulky residues in the vicinity of the CDR3, and the importance of intramolecular interactions between the CDR3 and the framework-2 region. Subsequently, we performed back-mutational analyses on the chimeric VHH. Back-mutations resulted in an increase of the thermal stability and affinity. These data suggested that back-mutations restored the intramolecular interactions, and proper positioning and/or dynamics of the CDR3, resulting in the gain of thermal stability and affinity. These observations revealed the molecular contribution of the framework region on VHHs and further designability of the framework region of VHHs without modifying the CDRs.

15. Repression of the PRELP gene is relieved by histone deacetylase inhibitors through acetylation of histone H2B lysine 5 in bladder cancer

Shozu K, Kaneko S, Shinkai N, Dozen A, Kosuge H, Nakakido M, Machino H, Takasawa K, Asada K, Komatsu M, Tsumoto K, Ohnuma SI, Hamamoto R.

Proline/arginine-rich end leucine-rich repeat protein (PRELP) is a member of the small leucine-rich proteoglycan family of extracellular matrix proteins, which is markedly suppressed in the majority of early-stage epithelial cancers and plays a role in regulating the epithelial-mesenchymal transition by altering cell-cell adhesion. Although PRELP is an important factor in the development and progression of bladder cancer, the mechanism of PRELP gene repression remains unclear. Here, we show that repression of PRELP mRNA expression in bladder cancer cells is alleviated by HDAC inhibitors (HDACi) through histone acetylation. Using ChIP-qPCR analysis, we found that acetylation of lysine residue 5 of histone H2B in the PRELP gene promoter region is a marker for the de-repression of PRELP expression. These results suggest a mechanism through which HDACi may partially regulate the function of PRELP to suppress the development and progression of bladder cancer. Some HDACi are already in clinical use, and the findings of this study provide a mechanistic basis for further investigation of HDACi-based therapeutic strategies.

16. Performance Comparison of Spectral Distance Calculation Methods

Oyama T, Suzuki S, Horiguchi Y, Yamane A, Akao K, Nagamori K, Tsumoto K.

Circular dichroism (CD) spectroscopy is a widely

used technique for assessing the higher-order structure (HOS) of biopharmaceuticals, including antibody drugs. Since the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use established quality control guidelines, objective evaluation of spectral similarity has been required in order to assess structural comparability. Several spectral distance quantification methods and weighting functions to increase sensitivity have been proposed, but not many reports have compared their performance for CD spectra. We constructed comparison sets that combine actual spectra and simulated noise and performed a comprehensive performance evaluation of each spectral distance calculation method and weighting function under conditions that consider spectral noise and fluctuations from pipetting errors. The results showed that using the Euclidean distance or Manhattan distance with Savitzky-Golay noise reduction is effective for spectral distance assessment. For the weighting function, it is preferable to combine the spectral intensity weighting function and the noise weighting function. In addition, the introduction of the external stimulus weighting function should be considered to improve the sensitivity. It is crucial to select the weighting function based on the balance between spectral changes and noise distributions for robust, sensitive antibody HOS similarity assessment.

Publications

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