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Antibody Dendrimers

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Supramolecular formations of antibodies by their specific molecular recognition to antigens are investigated. Linear and network supramolecular architectures have been constructed by using immunoglobulin G (IgG) and divalent or trivalent antigens, respectively. An amplification method of the detection signals for the target molecule in the biosensors based on the surface plasmon resonance (SPR) has been devised using the signal enhancement in the supramolecular assembly of the antibody with multivalent antigens. Novel dendritic supramolecular complexes are designed and prepared by using immunoglobulin M (IgM) or protein A/G as a core and IgGs as branches. One of the "antibody dendrimers" is composed of proteins with a molecular weight of about 2 million and constructed by non-covalent bonds. The dendrimer can bind antigens strongly with high specificity. The biosensor technique based on SPR shows that the antibody dendrimer has the advantage of amplification of detection signals for antigens.

Keywords. Antibodies, Biosensors, Non-covalent bond, Atomic force microscopy, Supramolecular chemistry

1 Introduction

In biological systems, life processes are led by the unique behavior of macromolecules such as proteins and DNAs. Molecular recognition by the macromolecules plays an especially important role, for example, in substrate specificities of enzyme and antigen–antibody reactions in human life. Selective recognition among macromolecules is achieved by non-covalent bonds with a large number of weak bonding interactions including hydrogen bonds, van der Waals, dipole and/or electrostatic interactions. We have focused our attention on the functions and structures of antibodies, because they can recognize larger and more complicated compounds with high specificity and efficiency than enzymes or synthetic host molecules. The immune system can selectively generate antibodies against virtually any molecule of interest. Antibodies are host proteins produced in response to the presence of foreign molecules in the body. Recently, with the advent of cell technology, it has become possible to prepare individual immunoglobulins called "monoclonal antibodies" in large amounts and in homogeneous form [1]. Monoclonal antibodies have become more and more important offering high possibilities as new chemical and tailor-made materials.

The basic structure of all antibody or immunoglobulin (Ig) molecules consists of four protein chains as shown in Fig. 1. The most common immunoglobulin class is IgG. Two identical heavy chains of approximately 50,000 Da and two identical light chains of about 25,000 Da are cross-linked each other by disulfide bonds. The molecule adopts a conformation that resembles the letter Y [2, 3]. There are two identical binding sites at the top of Fab fragments of IgG which are bound by flexible hinges with a single constant stem (Fc). The complementarity-determining regions in the antigen binding site differ in length and sequence between different antibodies and are mainly responsible for the specificity (recognition) and affinity (binding) of the antibodies to their target molecules. Fc does not bind the antigen, but it has other important biological activities, including the mediation of responses termed effector functions. Antibodies are divided into five classes: IgG, IgM, IgA, IgE, and IgD, on the basis of the number of Y-like units and the type of heavy-chain polypeptide they contain (Fig. 2). Each type has common characteristic sequences and variable sequences characteristic of antibodies in five types, respectively. IgM (Mw=960,000) has a pen-

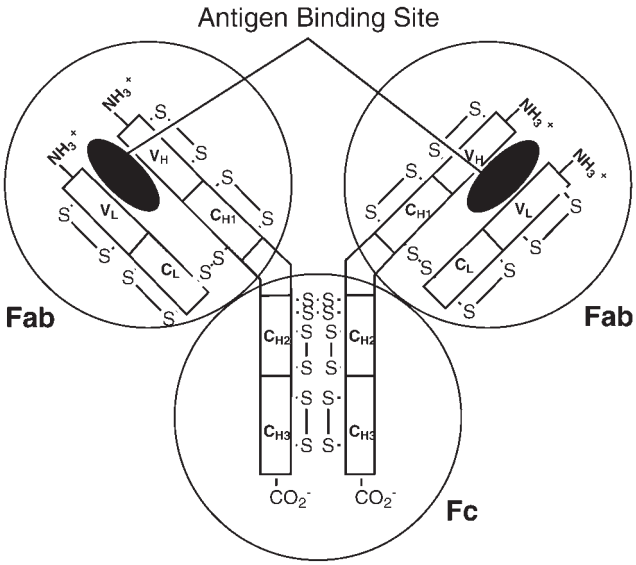


Fig. 1. The schematic representation of the structure of immunoglobulin G (IgG)

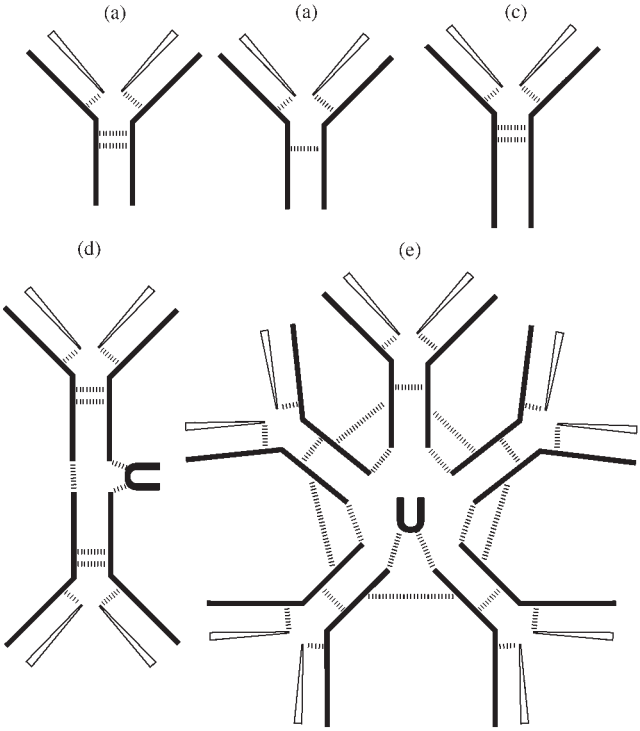


Fig. 2a–e. Structures of immunoglobulins. IgG (**a**), IgD (**b**), IgE (**c**), IgA (**d**), and IgM (**e**)

tameric structure of IgG and ten antigen binding sites in a single molecule [4]. The presence of ten antigen binding sites enables IgM to bind tightly to antigens containing multiple identical epitopes.

Recently, much attention has been directed toward antibodies not only in the field of biology but also in the field of chemistry because of their unique structures and functions. Antibodies, immunoglobulins, have been studied as sensors [5, 6], diagnostics [7, 8], DDS [9, 10], catalysts (catalytic antibodies) [11–13], and components for nanotechnology [14–16]. Antibodies especially have been widely used as efficient reagents to detect target molecules [17]. Based on the principle that an antibody reacts with an antigen specifically and by non-covalent bonds, several procedures have been developed in the immunosorbent assay. Labeled antibodies or antigens are used for the detection, localization, and quantification of biological constituents. More recently, an optical technique based on surface plasmon resonance (SPR) [18–22] or a microgravimetric quartz-crystal-microbalance (QCM) [23–25] technique has been found to be useful for measuring and characterizing macromolecular interactions in the increasingly expanding area of biosensor technology. SPR in particular has great potential for macromolecular interaction analysis in terms of sensitivity and signal translation. The use of biosensors based on SPR has made it possible to determine kinetic parameters in real time and without any labeling of biomacromolecules for detection. However, the SPR response reflects a change in mass concentration at the detector surface as molecules bind or dissociate; the specific sensing of substrates with low molecular weight is difficult. In such a case, functional molecules with a high molecular weight such as antibodies have a great potential for amplification of the response signals expressing molecular recognition event.

Here, we describe the design and preparation of antibody supramolecular complexes and their application to a highly sensitive detection method. The complex formation between antibodies (IgG) and multivalent antigens is investigated. When an antibody solution is mixed with divalent antigen, a linear or cyclic supramolecule forms [26–29]. With trivalent antigens, the antibody forms network structures. These supramolecular formations are utilized for the amplification of detection signals on the biosensor techniques.

Sensitivity and specificity are required and important in the construction of excellent detection methods. IgG is generated in a final stage of immunization, so it is matured and highly selective. IgM has a pentameric structure of IgG and ten antigen binding sites in a single molecule. It binds large multivalent antigens strongly (avidity) because of their multivalent structures. However, IgM is the first class of antibody to appear in the serum after exposure to an antigen; it is unmatured and less specific for the antigen than IgG. In order to design an antibody system with a high specificity and a high affinity, a combination of the functions of both IgG and IgM seems to be important. We design dendritic antibody supramolecules as artificial antibodies.

2
Experimental Section

2.1
Materials

2.1.1
Anti-Viologen Antibodies

Monoclonal antibodies have been elicited for the viologen derivative, 4,4′-bipyridinium 1-(carboxypentyl)-1′-methyl-dichloride (**1**) (Fig. 3a). The hapten **1** was coupled to keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) using carbonyldiimidazole (CDI). KLH-**1** and BSA-**1** were purified by size exclusion chromatography using Sephadex G-150 and used as an antigen for the immunization to mice and immunoassay, respectively. Balb/c mice aged 8 weeks were immunized with KLH-**1** emulsified in Freund's complete adjuvant 6 times at weekly intervals. Three days after the final injection (boost), spleen cells were removed and used for the fusion experiments. Spleen cells from a mouse were fused with the SP 2/0 mouse myeloma cells [30]. The hybridomas secreting antibodies for viologen were detected by enzyme-linked immunosorbent assay (ELISA). The tissue culture supernatants were added onto the ELISA plate coated with 0.3 mg mL^{-1} BSA-**1** and incubated 90 min. The amount of antibody bound to the antigen was measured using goat anti-mouse immunoglobulins labeled with alkaline phosphatase. Hybridomas secreting anti-viologen antibodies were cloned two times by limiting dilution. The ascites fluid was harvested after 10 days. The monoclonal antibodies were purified by affinity chromatography using protein A (Amersham Ampure PA kit). The purity of antibodies was checked by SDS-PAGE electrophoresis (Phast System, Pharmacia LKB, Uppsala, Sweden). Antibody solutions were stored and treated in 0.1 M phosphate borate buffer (pH 9.0).

2.1.2
Anti-Porphyrin Antibodies

A monoclonal antibody (IgM) for a cationic porphyrin has been prepared using [5-(4-carboxyphenyl)-10,15,20-tris-(4-methylpyridyl)]porphine (3MPy1C) as a hapten [31]. IgG specific for anionic porphyrin, *meso*-tetrakis(4-carboxyphenyl)porphine (TCPP), has been prepared [32, 33]. The cationic porphyrin, 3MPy1C has been attached to the IgG via activation of carboxylic acid in 3MPy1C using the condensation agent, carbonyldiimidazole. The IgG-cationic porphyrin conjugate was purified by column chromatography using Sephadex G-150 to remove the porphyrins that did not react with the antibody.

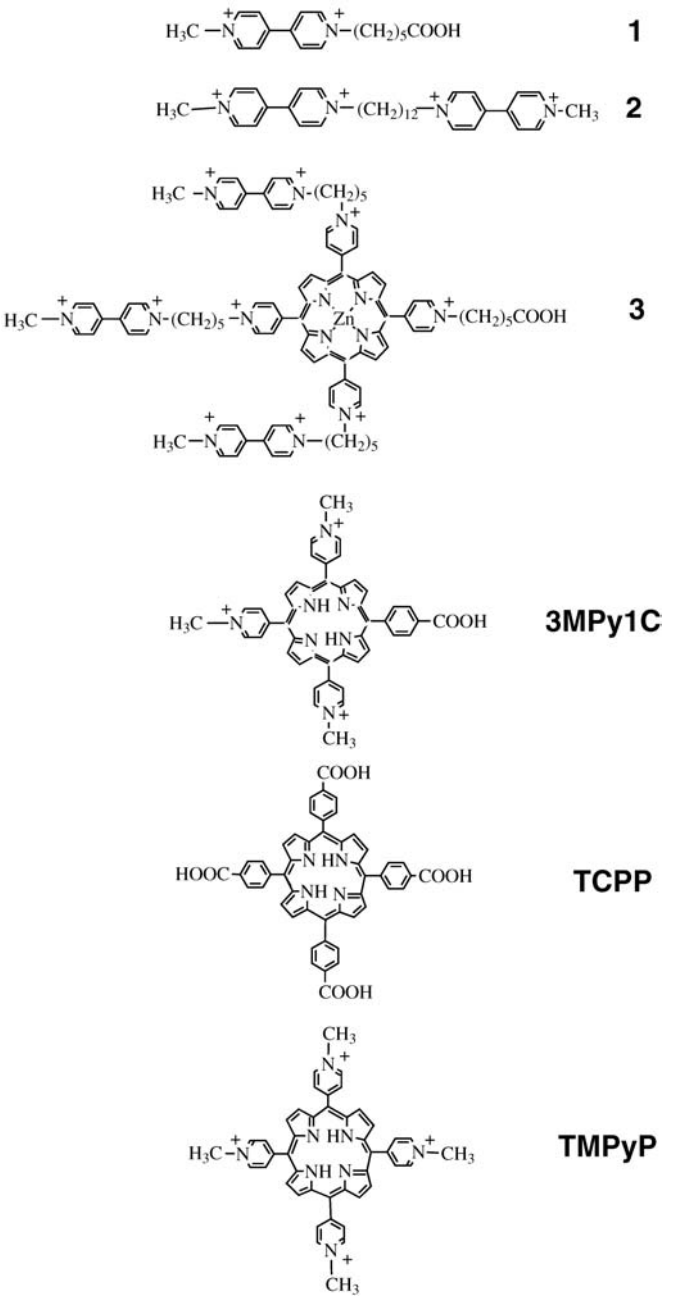


Fig. 3. Haptens and multivalent antigens prepared in this syudy. The viologen derivative, 4,4′-bipyridinium, 1-(carboxypentyl)-1′-methyl-dichloride (**1**), divalent antigen **2**, and trivalent antigen **3**. Anti-porphyrin antibodies were elicited for [5-(4-carboxyphenyl)-10,15,20-tris-(4-methylpyridyl)]porphine (**3MPy1C**) or *meso*-tetrakis(4-carboxyphenyl)porphine (TCPP). *meso*-Tetrakis(4-methylpyridyl)porphine (**TMPyP**) was used to investigate the specificity of the antibody dendrimer for porphyrins

2.2 Measurements

2.2.1 *Competition ELISA*

The antibody solution (1.6×10^{-8} M) and substrate solutions with various concentration from 10^{-9} M to 10^{-3} M were mixed on a BSA-coated plate. The mixed solution of antibodies and substrates was allowed to stand for 1 day at room temperature, and then transported to the ELISA plates pre-coated with BSA-hapten and BSA blocking buffer. Absorbance at 405 nm for the resulting enzymatic hydrolysis product (*p*-nitrophenolate) by alkalinephosphatase of the second antibody was recorded on an Immuno-Mini NJ-2300 to determine the amount of antibody bound to BSA–hapten.

2.2.2 *Biosensor Technique*

Biospecific interaction analysis (BIA) was performed by using a BIAcore X system (BIACORE, Uppsala, Sweden) based on the surface plasmon resonance. One of the anti-viologen antibodies was immobilized on the sensor chip by activating carboxymethyl groups on the surface matrix by a mixture of *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N′*-(dimethylaminopropyl)-carbodiimide (EDC). The response signal (RU) of the BIAcore apparatus is proportional to the change in the refractive index at the surface and is generally assumed to be proportional to the mass of substance bound to the chip. Kinetics of association to the immobilized antibody were studied by measuring the amount of bound substrate as a function of time when a solution containing viologen derivative or antibody passes over the chip surface. The dissociation kinetics were monitored by detecting the time-dependence of the mass decrease after the surface was subsequently washed with buffer.

2.2.3 *AFM Measurement*

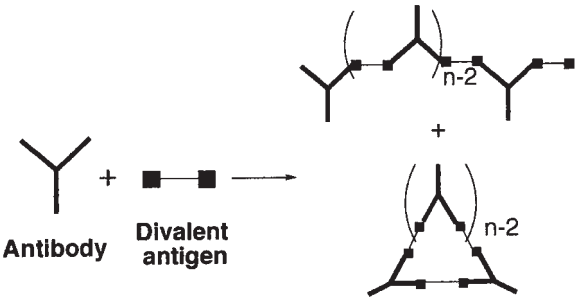
A total of 2 µL of the antibodies or the antibody-supramolecules (0.5 mg mL^{-1} in 0.1 M phosphate borate buffer, pH 9.0) solutions was used for the experiment. These samples were air-dried onto the surface of freshly cleaved HOPG (highly oriented pyrolytic graphite, 1.2 cm×1.2 cm) substrate. The sample was allowed to stand for 1 day in a desiccator with $CaCl_2$ to dry the sample before it was transferred into the AFM. The sample surface was observed by contact AFM under mild conditions such that any damage caused by, for example, the preparation was minimized [34]. AFM measurements were carried out by using a Si_3N_4 tip with a radius of approximately 40 nm. All measurements were taken on a multimode Nanoscope IIIa (Digital Instruments, Santa Barbara, CA). The line scan speed was 2 Hz with 512 pixels per line. All scales were calibrated against a standard sample (5 µm×5 µm grid) and rechecked with graphite.

3
Supramolecular Formation of Antibodies with Multivalent Antigens

In this study, methyl viologen is selected as one of the target molecules to be detected. Although viologens are harmful, they are well-known functional molecules which act as herbicides and electron acceptors. Methyl viologen has been suggested as a potential etiologic factor in Parkinson's disease because of the structural similarity to the known dopaminergic neurotoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [35, 36]. Anti-viologen antibodies [37–40] may be expected to be useful not only as a highly sensitive reagent of viologens but also as a functional material to control the electron transfer from electron donors to viologens. It is important to use the anti-viologen antibodies for the sensitive and specific detection of viologens by the SPR biosensor because methyl viologen is a charged substrate with low molecular weight. However, the detection of viologens at low concentrations is difficult owing to the low sensitivity (small response) in a common SPR biosensor technique using corresponding antibodies. To improve the sensitivity, it is important to detect methyl viologen as a large response signal caused by the antibody bindings. A solution to this problem is thought to be the inhibition of the supramolecular formation between the anti-viologen antibody and viologen dimer by methyl viologen. We investigated the complex formation of one of the antibodies, 10D5, with methyl viologen or viologen dimer **2** and trivalent antigen **3** using the SPR biosensor.

3.1
Supramolecular Formation of Antibodies with Divalent Antigens

The specific binding of the antibody (divalent) and antigen dimer (divalent) produces the supramolecules such as linear or cyclic antigen–antibody oligomers as shown in Scheme 1 [26, 27]. Stepwise additions of antigen dimer and antibody to the sensor chip of the biosensor, whose surface is modified with the antibody, leads to the enhancement of the response signal intensity in SPR due to the formation of antigen–antibody supramolecules. When methyl viologen is added to the flow cell instead of viologen dimer, the binding sites of the



Scheme 1. Complex formation of antibodies (IgG) with divalent antigens

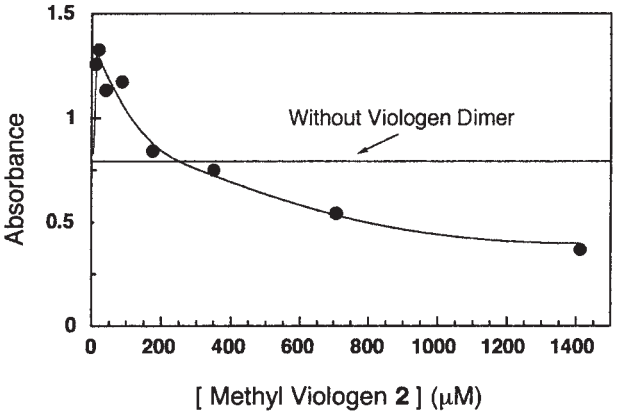


Fig. 4. Competition ELISA between antibody 10D5 and viologen dimer **2**. A number of antibody molecules are immobilized on the ELISA plate at lower concentrations of viologen dimer **2**

antibody at the end of the supramolecule are occupied by methyl viologen and cannot bind viologen dimer that acts as a connector of the antibodies in the supramolecular formation. The addition of methyl viologen may cause a reduction in the response signal enhancement. In this method, the binding of methyl viologen to the antibody affects the next growing step in the supramolecular formation between the antibody and viologen dimer. The amount of methyl viologen (Mw=257) is expressed as the amount of the antibody (Mw=150,000) that cannot form the supramolecules between the viologen dimer and the antibody.

The antibody 10D5 (IgG_1) binds hapten **1** with the dissociation constant K_d=2.0×10^{-7} M. The dissociation constant between antibody 10D5 and methyl viologen was found to be 2.0×10^{-7} M. The antibody 10D5 recognizes the bipyridinium moiety with high specificity. Figure 4 shows the result of competition ELISA between antibody 10D5 and viologen dimer **2**. At low concentrations of viologen dimer, the absorbance of the product of enzymatic reactions is higher than that in the absence of viologen dimer. It is suggested that a number of antibody molecules are immobilized to the ELISA plate in the presence of viologen dimer. The signal intensity on the SPR biosensor also increased on the addition of an aqueous solution of antibody 10D5 to the sensor chip on which the viologen dimer–antibody complex was pre-coated. Figure 5 shows the sensorgram of the repeated injection of the aqueous viologen dimer and antibody solutions. The signal intensity enhanced by the binding of antibody to the viologen dimer–antibody complex. The viologen dimer molecule is considered to act as a connector between antibodies. It is suggested that the phenomena observed in the ELISA and SPR measurements are ascribable to the higher-order complex formation between antibodies and viologen dimer.

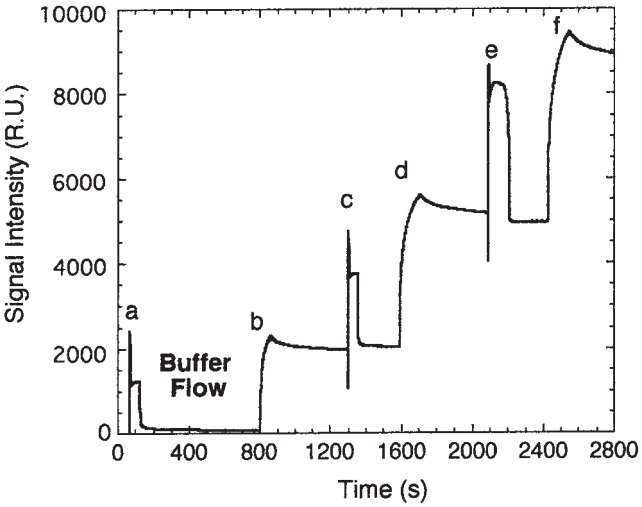
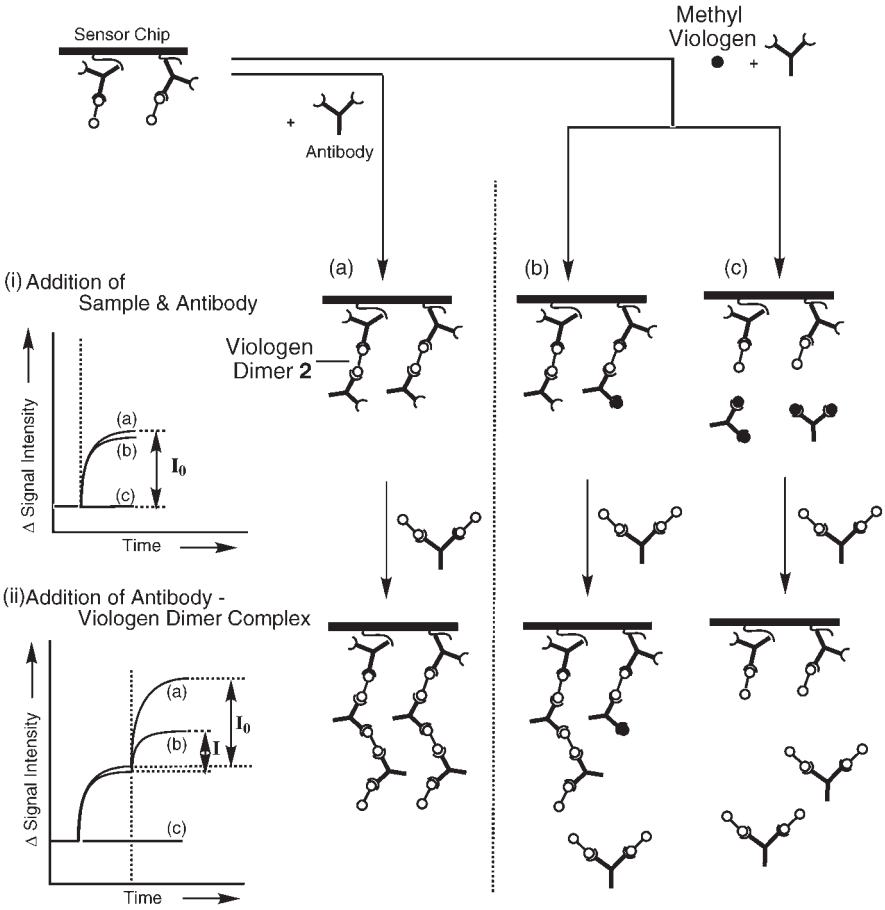


Fig. 5a–f. The sensorgram of the repeated injection of the aqueous viologen dimer **2** (**a**, **c**, **e**) and the antibody (**b**, **d**, **f**) solutions. [Viologen dimer 2]=2.0 μM and [antibody]=2.0 μM in phosphate borate buffer. Injection period 60 s for **a**–**c** and 120 s for **d**–**f**. A solution of viologen dimer **2** or the antibody passes over the surface of the sensor chip for 60 or 120 s at a constant flow rate of 20 μL min^{-1}. The surface of the sensor chip was subsequently washed with buffer

3.2
Applications for Highly-Sensitive Detection Method of Small Molecules by the Supramolecular Complexes between Antibodies and Multivalent Antigens

We found that the supramolecular formation of antibodies and viologen dimer **2** produces an SPR signal enhancement of the biosensor. The additional binding of the antibody to the viologen dimer–antibody complex gives a remarkable increase of signal intensities. On the other hand, the addition of methyl viologen (viologen monomer) instead of viologen dimer is expected to block the antigen binding sites and to inhibit the additional antibody binding. A small amount of methyl viologen can be detected as a decrease of signal enhancement due to the inhibition of the complex formation between viologen dimer and antibody by methyl viologen, compared with the signal intensity of complete supramolecular formation between the antibody and viologen dimer. Scheme 2 shows the strategy for the amplification of detection signals for methyl viologen based on the signal enhancement by the supramolecular formation between the antibody and viologen dimer on the biosensor technique. This system includes a two-step procedure as follows: (i) the injection of the aqueous solution of anti-viologen antibody with methyl viologen to the sensor chip whose surface is modified with the antibody–viologen dimer complex, and then (ii) the addition of antibody–viologen dimer (1:2) complex to the previous state. The changes of the signal intensities in the presence of methyl viologen are compared with that in the absence of methyl viologen.

The amount of antibody immobilized on the sensor chip decreased with increasing concentration of methyl viologen. To enlarge the difference in the sig-



Scheme 2. Strategy for the highly sensitive detection of viologen based on the SPR biosensor technique. Inhibition of the complex formation of the antibody with viologen dimer by methyl viologen and the signal enhancement due to the supramolecular formation between the antibody and viologen dimer. Antibody 10D5 viologen dimer complex is immobilized onto the surface of the sensor chip. An aqueous solution of antibody 10D5 and a sample including methyl viologen or that without methyl viologen is injected into the flow cell (*i*) before the addition of the complex between antibody 10D5 and viologen dimer **2** (*ii*). The supramolecular formation between antibody 10D5 and viologen dimer **2** without methyl viologen (**a**), and that in the presence methyl viologen (**b**) and (**c**). [Methyl viologen] <[antibody combining site]: (**b**) and [methyl viologen] >>[antibody combining site]: (**c**)

nal intensities in the presence of a small amount of methyl viologen, a solution of the antibody–viologen dimer complex was added to the previous state (i). Figure 6a shows the differences in the response signal intensities (I_0–I) between the complete supramolecular system in the absence of methyl viologen (I_0) and that in the presence of various concentrations of methyl viologen (I) at each step. The differences in signal intensities due to the binding of the additional antibody (0.9 μM) in the presence of methyl viologen ranging the concentration

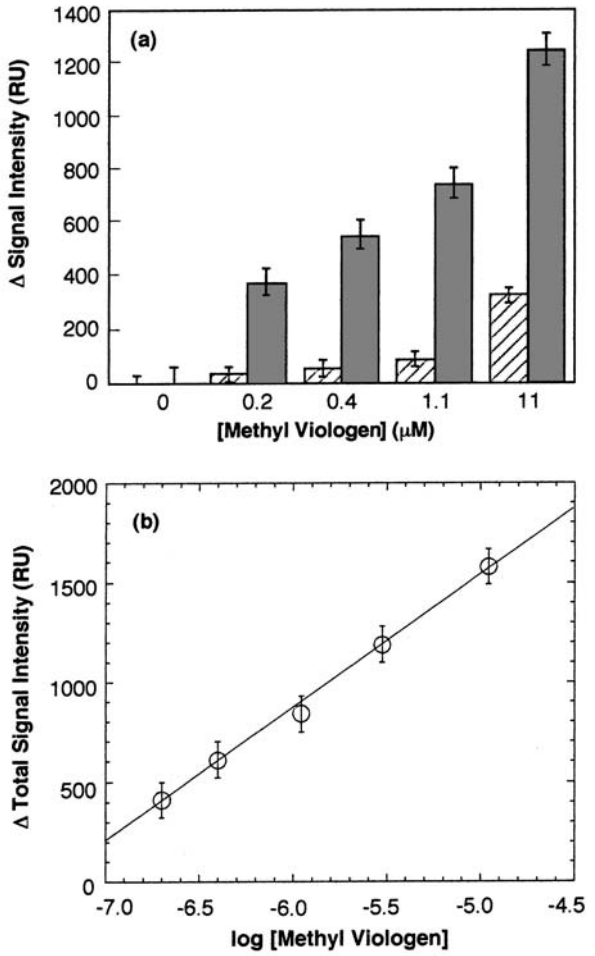


Fig. 6a,b. The differences in the response signal intensities between the complete supramolecular system in the absence of methyl viologen and that in the presence of various concentrations of methyl viologen at each step. **a** Changes in the signal intensities by the addition of an aqueous solution of antibody 10D5 and methyl viologen in the step (i) (*left side*) and those by the addition of the solution including the antibody–viologen dimer complex in the step (ii) (*right side*). **b** The relationship between total changes in the signal intensities and the concentration of methyl viologen (logarithm)

from 0.2 to 1.1 μM is slight in the step (i). However, further addition of viologen dimer **2** and antibody solutions in the step (ii) causes a clear difference in the response signal intensities in the same concentration range of methyl viologen. The total changes in the signal intensities were found to have a linear relationship with the logarithm of the concentration of methyl viologen as shown in Fig. 6b. The sensitivity in this system is 140-fold larger than that in the simple addition of methyl viologen to the antibody immobilized on the surface of the sensor chip. It is clear that this system can be utilized for the quantitative detec-

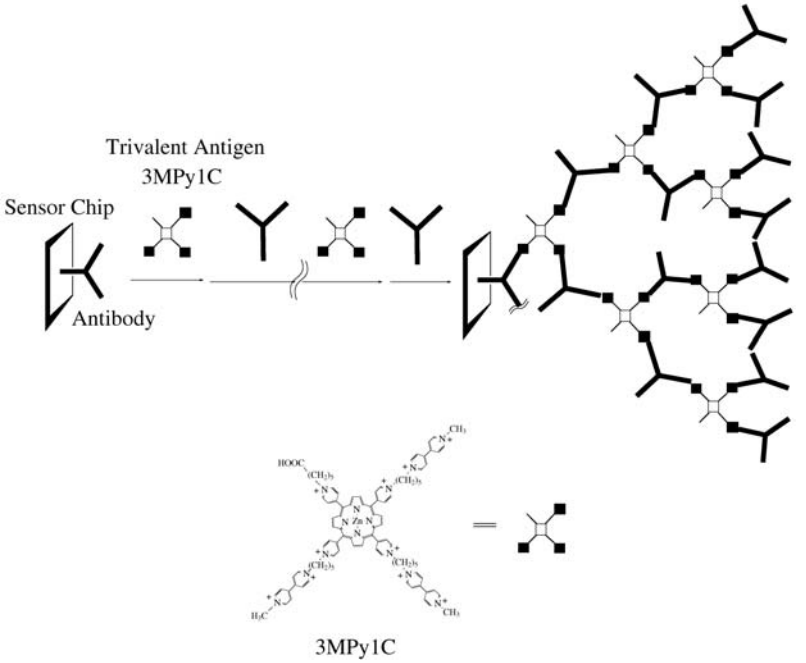


Fig. 7. A proposed structure of the complex of the antibody with the trivalent antigen immobilized on the surface of the sensor chip

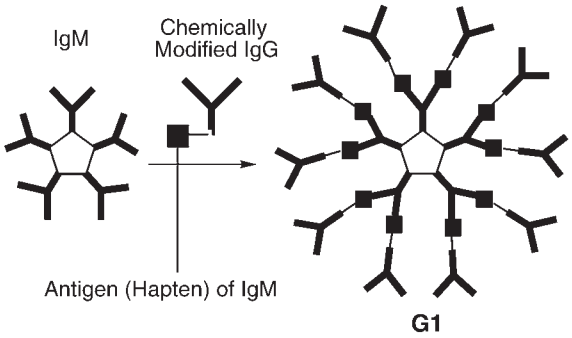
tion of methyl viologen. Amplification of methyl viologen sensing processes is realized by the inhibition of complex formation between the antibody and viologen dimer–antibody complex and signal enhancement due to the supramolecular formation of the antibody and viologen dimer.

In the case of trivalent antigen **3**, the signal intensities on SPR biosensors increased by the addition of the antibody in the presence of the trivalent antigen were twice that in the presence of the divalent antigen. Figure 7 shows the schematic representation of the complex formation between the antibody and the trivalent antigen immobilized onto the surface of the sensor chip. AFM images of the complex obtained from the homogeneous solution of the mixture also suggested the presence of the network structures.

4
Dendrimers Constructed by IgM and Chemically Modified IgG

4.1
Preparation of Antibody Dendrimers and their Topological Structures

A novel antibody supramolecule is designed and prepared by using immunoglobulin M (IgM) as a core and chemically modified IgGs as branches as shown in Scheme 3. The characteristic binding ability and specificity of IgG were found to remain during the chemical modification of IgG with 3MPy1C. When IgM for



Scheme 3. The synthetic route of the complete antibody dendrimers. An ideal structure of the dendritic supramolecule is shown as **G1**

3MPy1C is treated with IgG covalently bound cationic porphyrin, IgM binds the cationic porphyrin attached on the IgG to give a dendritic antibody supramolecule "antibody dendrimer" (**G1** in Scheme 3). The dendritic supramolecules are composed of proteins with molecular weight of about 2 million and constructed from non-covalent bonds. The structural observation of the antibody dendrimer was carried out by using AFM. At first, an individual antibody molecule, IgG or IgM, was observed by AFM at room temperature depositing from a solution onto a freshly cleaved HOPG surface. Figure 8 shows AFM images of the IgG molecule whose molecular weight is 150,000. Characteristic T or Y shape molecules can be seen [41]. The overall lateral dimension is 40–50 nm as shown A, B, and C in Fig. 8, which is in good agreement with the expected values [42]. Although these molecules are monoclonal antibodies, each antibody molecular image is not identical. There are two fragments with the same height and one fragment with different height with the other two fragments in each molecular image. Fab fragments and an Fc fragment can be differentiated by comparison of the height among three fragments. The angles between the Fab fractions of each immunoglobulin are different. Each antibody molecules takes a somewhat

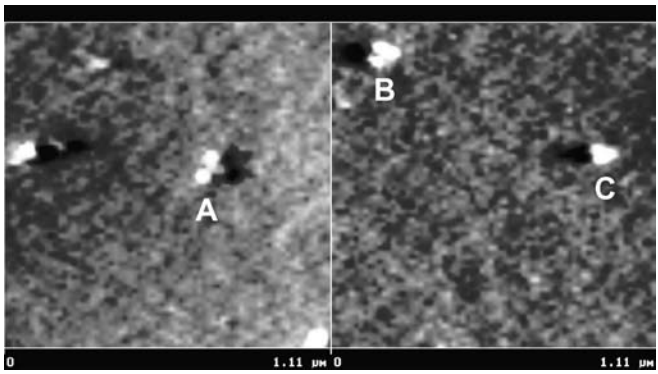


Fig. 8. AFM images of the individual antibody (IgG) molecules

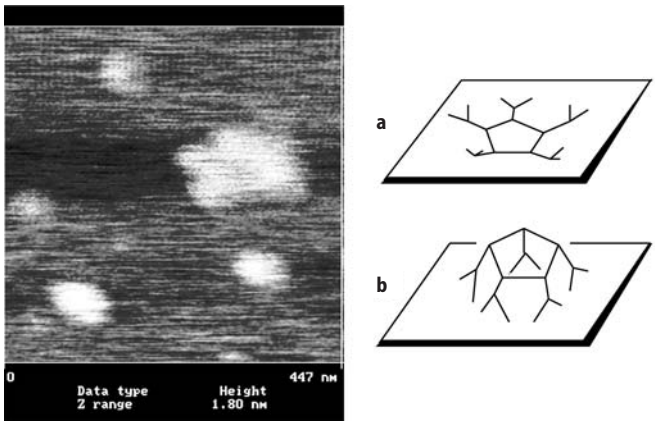


Fig. 9a,b. The molecular images of the monoclonal IgM on a cleaved highly orientated pyrolytic graphite (HOPG) surface and schematic representation of the images for IgM. A flat pentagram (**a**) and a smaller object with higher center (**b**)

different shape. This is probably due to the flexibility of its hinge region. Figure 9 shows IgM images. Two kinds of images are evident, although the object is a single species. The bigger one is a flat pentamer and the smaller one has a higher center. The results are consistent with those obtained by the cryo-AFM measurement [43, 44]. The sample surface was observed under conditions such that any damage caused by scanning the cantilever is minimized and that any nonspecific assembly among antibodies does not occur. Figure 10 shows an AFM image of the dendrimer. The image of the dendrimer was twice as large as that of starting IgM. Some branches (IgGs) can be seen outside of the IgM core. Such an assembled structure was not observed in a chemically modified IgG solution or an IgM solution alone.

The similar antibody dendrimers are prepared by the other route as shown in Scheme 4. The building block used as a branch was prepared by the specific

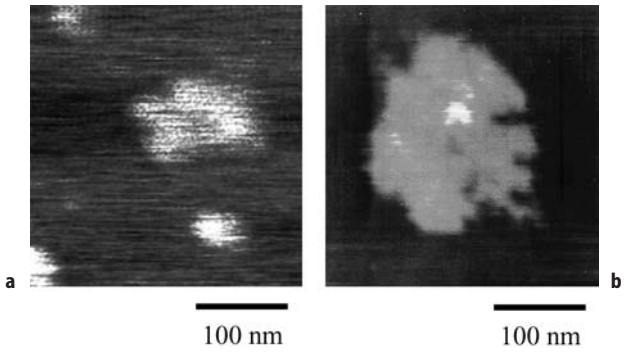
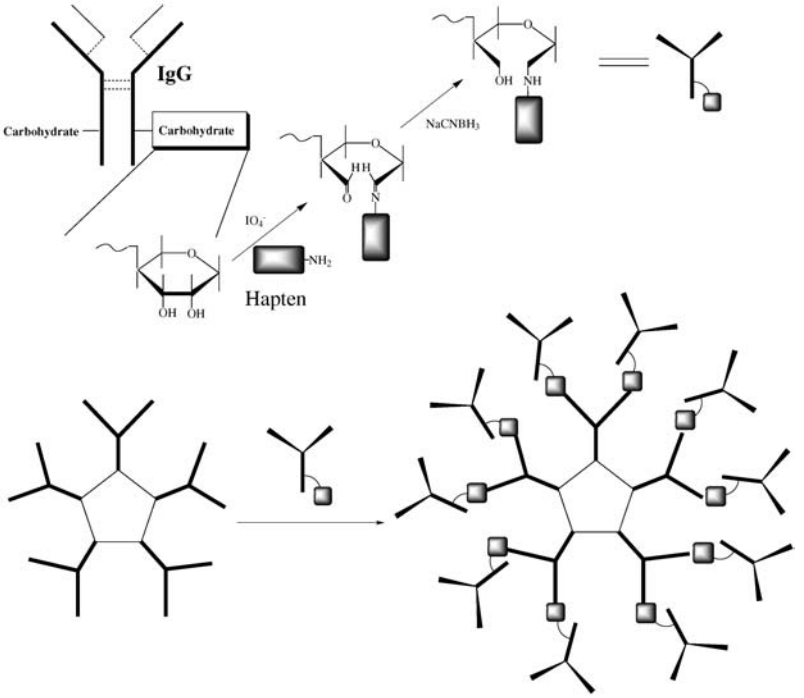


Fig. 10a,b. The AFM images of IgM (**a**) and the antibody dendrimer (**b**). A total of 2 μL of solutions of the antibodies (3.0×10^{-9} M) in 0.1 M phosphate borate buffer (pH 9.0) was deposited onto the surface of highly oriented pyrolytic graphite (HOPG) and air-dried



Scheme 4. Antibody dendrimers prepared by the combination of IgM with IgG attached hapten to the carbohydrate moiety in the Fc fragment

modification of a hapten to the carbohydrate moiety in the Fc fragment of the antibody molecule. Site-specific modification of antibody molecule makes it possible to prepare a homogeneous building block rather than amine coupling of the hapten to the antibody molecule. The combination of IgM and IgGs attached hapten to the carbohydrate part leads to the construction of a complete radial structure that possess antigen binding sites on the surface of the antibody dendrimer.

4.2 Binding Properties of Antibody Dendrimers for Antigens

The binding property of the antibody dendrimer (**G1**) with a cationic or anionic porphyrin was measured by the enzyme-linked immunosorbent assay (ELISA). Figure 11a shows the binding properties of the IgG, IgM, and **G1** with the cationic porphyrin, *meso*-tetrakis(4-methylpyridyl)porphine (TMPyP). Although IgG did not bind the cationic porphyrin and IgM bound the cationic porphyrin, the dendrimer did not bind TMPyP. These results show that the cationic porphyrin attached to IgG occupies the binding sites of IgM in the dendrimer, thus there are no free binding sites against TMPyP on IgM. Figure 11b shows the binding of IgG, IgM, and **G1** to TCPP. The IgM used in this study can bind both anionic

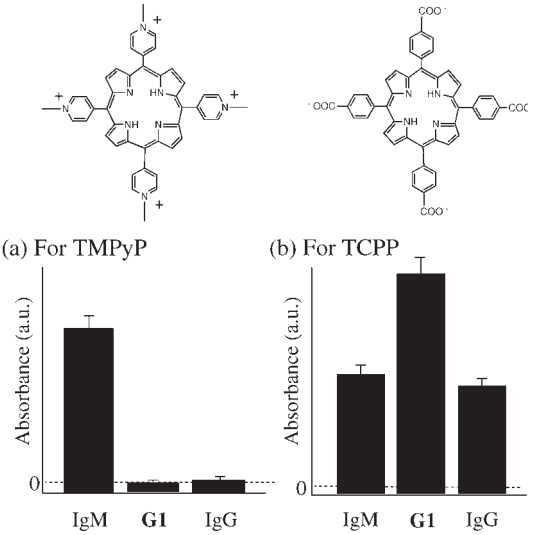


Fig. 11a,b. Binding affinities of IgG, IgM, and the antibody dendrimer (**G1**) with the cationic porphyrin (TMPyP) (**a**) and those with the anionic porphyrin (TCPP) (**b**) estimated by ELISA

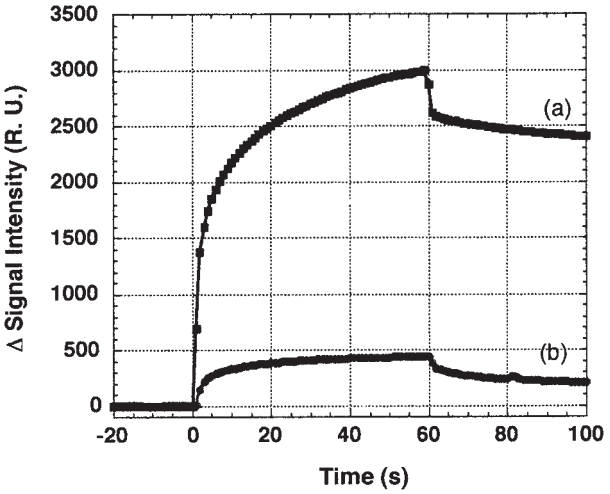


Fig. 12a,b. The sensorgrams for the binding of the antibody dendrimer (**a**) or IgG (**b**) to the anionic porphyrin immobilized onto the surface of the sensor chip. Phosphate borate buffer (0.1 M, pH 9.0) was used. TCPP was immobilized via hexamethylenediamine spacer onto the sensor chip and then a solution of IgG or the dendrimer was injected to the flow cell. After 60 s from the injection of the antibody solutions, flow cell was filled with buffer

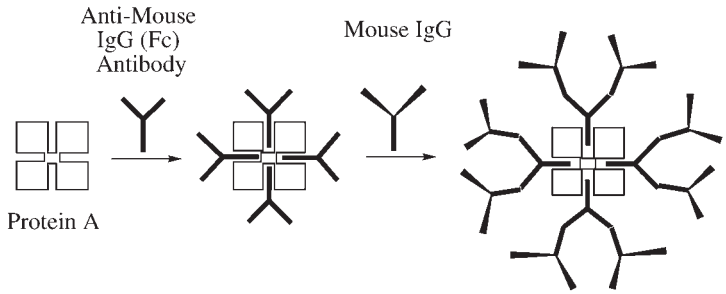
and cationic porphyrins, due to the low specificity of IgM against porphyrins. Both IgM and IgG bound TCPP, while **G1** bound TCPP more efficiently than IgM or IgG. The increase in affinity of **G1** for the anionic porphyrin indicates that many IgG molecules attach to the surface of the IgM molecule.

The biosensor technique based on surface plasmon resonance (SPR) [19] shows that the antibody dendrimer allows an advantageous amplification of the detection signals for antigens. A solution of **G1** was added to the sensor chip on which TCPP was pre-coated by the coupling with hexamethylenediamine as a spacer. The total concentration of the antibody was fixed at 0.2 mg mL^{-1} (0.1 mg of IgM+0.1 mg of chemically modified IgG in 1 ml buffer for **G1**). The sensorgram for the binding of the antibody dendrimer to TCPP was compared with that of IgG to TCPP as shown in Fig. 12. The signal intensity increased by the injection of the antibody dendrimer was sufficiently larger than that of simple addition of IgG. Taking into account the change of the binding property of the antibody dendrimer for porphyrins with the increase in the amount of bound antibody to the anionic porphyrin on the SPR biosensor, the antibody dendrimer has many IgG molecules successively bound to IgM molecule.

5
Other Methods for the Construction of Antibody Dendrimers

Antibody dendrimers were prepared by using proteins with high affinity for the Fc fragment in IgG as a core and IgG as a branch. In this procedure, an IgM-like complex can be constructed without any modification of IgG. Staphylococcal protein A or streptococcal protein G were examined as a core component of the antibody dendrimer. Protein A or G plays an important role in molecular biology owing to its specific interaction with the Fc portion of immunoglobulins. Many immunological methods have been developed and refined using these proteins as a reagent, including immunoprecipitation techniques and double sandwich immunoassays. In addition, solid-phase protein A or G has been widely used as a carrier in affinity chromatography for the purification of antibodies. The protein A molecule consists of four highly homogeneous Fc-binding domains. Therefore, protein A is thought to be a suitable component of the core of the dendrimer. The antibody dendrimer of protein A with IgG was prepared according to Scheme 5. The first generation of the dendrimer was prepared by the addition of mouse IgG molecules to protein A. The second-generation dendrimer was prepared by using goat IgG specific for Fc of the mouse IgG antibody developed in goat and mouse IgG specific for the target molecule. Each dendrimer was applied for the detection of the antigens on SPR biosensors. The signal intensities of the binding of the antigen immobilized on the surface of the sensor chip increased in proportion to the number of the generation of the dendrimer as shown in Fig. 13.

The immobilization of the hyperbranched spherical structures onto physical transducers greatly increases the binding capacity of the surface and leads to enhanced sensitivity and extended linearity of biosensors. Nucleic acid dendrimers were prepared and their amplification properties for the detection of DNA were examined using mass-sensitive transducers [45, 46]. Antibodies



Scheme 5. Preparation of the dendritic supramolecules with protein A as a core and IgGs as branches

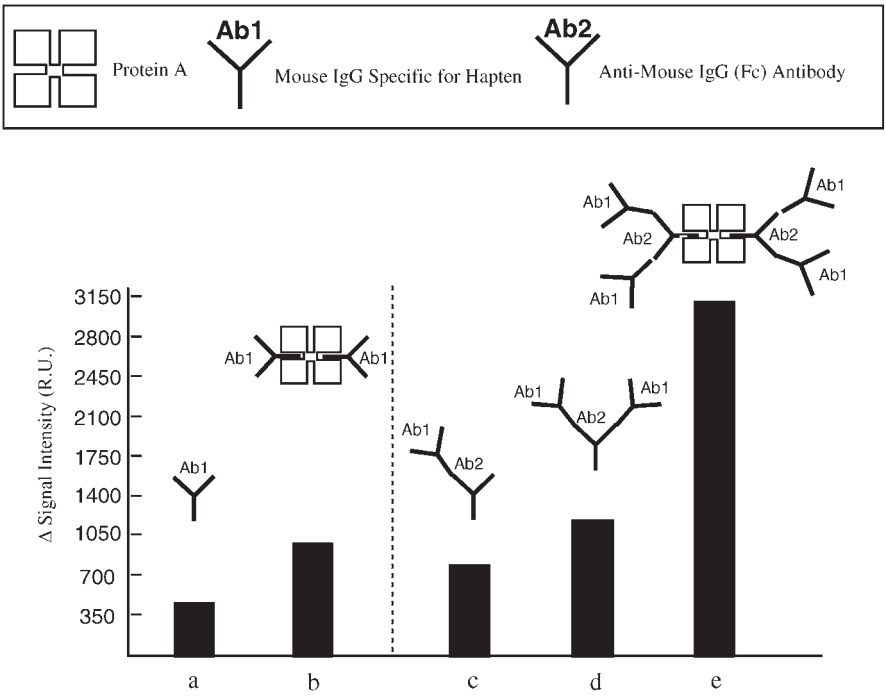
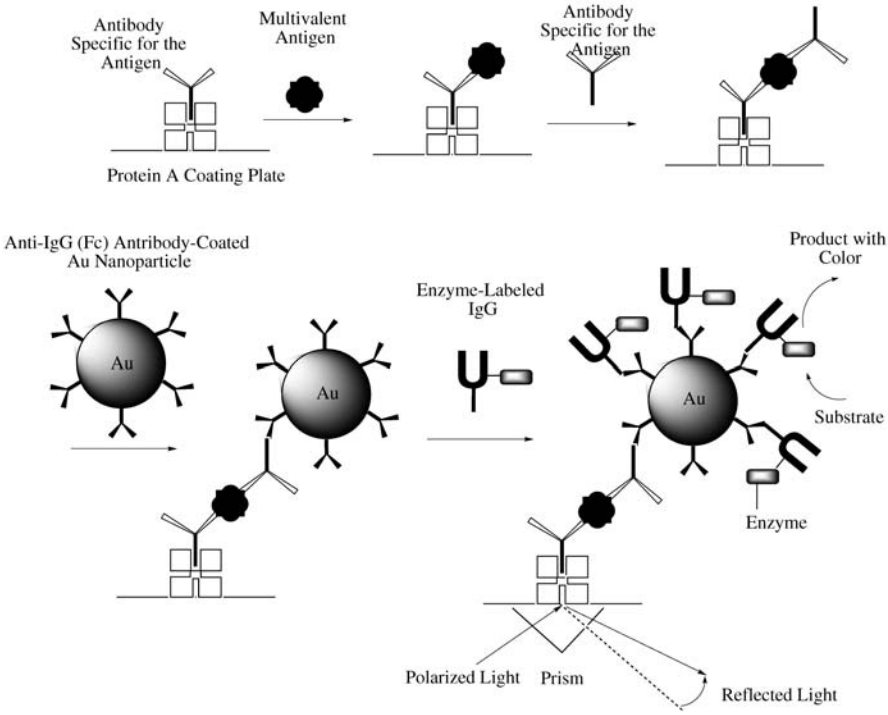


Fig. 13a–e. The increase of the signal intensities by the addition of the dendritic complexes composed of IgGs and protein A. The hapten was immobilized to the surface of the SPR sensor chip. The increase of the signal intensities on the complex formation of hapten with the antibodies were monitored. The addition of mouse IgG specific for hapten (**Ab1**) (**a**), the complex of the **Ab1** with protein A (**b**), one to one complex of **Ab1** with anti-mouse IgG (Fc) antibody (**Ab2**) (**c**), two to one complex of **Ab1** with **Ab2** (**d**), and two to one complex of **Ab1** with **Ab2** in the presence of protein A (**e**)



Scheme 6. A highly sensitive detection method using an antibody specific for IgG (Fc) and gold nanoparticle

attached to the surface of gold nanoparticles were used for the organization and patterning of inorganic nanoparticles into two- and three-dimensional functional structures [47]. These materials have a potential as chemical, optical, magnetic, and electronic devices with useful properties. Taking account the advantage of these materials, we propose a novel highly sensitive detection method of a multivalent target molecule as shown in Scheme 6. The combination of SPR biosensor technique and enzyme-linked immunosorbent assay is utilized for the detection of a small amount of target molecule. Anti-IgG (Fc) antibodies attached to the surface of gold nanoparticles can bind the antibody (IgG) bound to the target molecule immobilized onto the surface of the sensor chip and the antibodies labeled with enzymes. The detection signals are expressed as a color of the enzymatic reaction products and also as an enhancement of the SPR.

6 Conclusions

An enhancement of SPR signal intensity was observed by the addition of the antibody to the divalent antigen–antibody complex immobilized onto the surface of the sensor chip, indicating the formation of linear supramolecules. An amplification method of the detection signals for a target molecule has been

devised by using the signal enhancement in the supramolecular assembly of anti-viologen antibodies and divalent antigens. Target substrate added to the flow cell of SPR can be detected quantitatively by monitoring the total amount of the antibody bound to the surface of the sensor chip. The sensitivity of this system was found to be two orders larger than that of the simple addition of target substrate to the antibody immobilized on the surface of the sensor chip. This method can be potentially applied to many compounds with a highly sensitivity and specificity by using corresponding antibodies and dimers of the target molecule (divalent antigen). A trivalent antigen formed a dendritic network structure and enhanced the response signal intensities on the SPR biosensor.

New antibody dendrimers were designed and prepared by the combination of IgG and IgM, that is, using IgM as a core and IgG as branches. Many binding sites of IgG were arranged radially on the surface of one object; the resulting artificial antibodies bound antigens more selectively than IgM and more strongly than IgG. The characteristic features of the antibody dendrimer are: (i) they are composed of proteins, (ii) have a large size with molecular weight of about 2 million, (iii) are constructed from non-covalent bonds, and (iv) bind antigen strongly with high specificity. The antibody dendrimer will be used as functionalized materials for sensitive detection of many kinds of chemicals, for diagnosis, and for drug delivery systems. The other antibody dendrimers are prepared by the specific modification of a hapten to the carbohydrate of antibody molecules. Antibody dendrimers consisting of protein A or G and IgG are also prepared. They are found to be useful as a functional material for the amplification of detection signals of target molecules in SPR sensors.

7 References

1. Kohler G, Milstein C (1975) Nature 256:495
2. Terry WD, Matthews BW, Davies DR (1968) Nature 220:239
3. Silverton EW, Navia MA, Davies DR (1977) Proc Natl Acad Sci USA 74:5140
4. Tomalia DA, Naylor AM, Goggard III WA (1990) Angew Chem Int Ed Engl 29:138
5. McDonnell JM (2001) Curr Opin Chem Biol 5:572
6. Scheller FW, Wollenberger U, Warsinke A, Lisdat F (2001) Curr Opin Biotechnol 12:35
7. Borrebaeck CAK (2000) Immunol Today 21:379
8. Stoll D, Templin MF, Schrenk M, Traub PC, Vöhringer CF, Joos TO (2002) Front Biosci 7:C13
9. Kaneda Y (2000) Adv Drug Deliv Rev 43:197
10. Garnett MC (2001) Adv Drug Deliv Rev 53:171
11. Golinelli-Pimpaneau B (2000) Curr Opin Struct Biol 10:697
12. Feiters MC, Rowan AE, Nolte RJM (2000) Chem Soc Rev 29:375
13. Lu Y, Berry SM, Pfister T D (2001) Chem Rev 101:3047
14. Shenton W, Davies SA, Mann S (1999) Adv Mater 11:449
15. Harada A, Yamaguchi H, Okamoto K, Fukushima H, Shiotsuki K, Kamachi M (1999) Photochem Photobiol 70:298
16. Yamaguchi H, Kamachi M, Harada A (2000) Angew Chem Int Ed 39:3829
17. Yamaguchi H, Harada A, Kamachi M (1998) Reactive Functional Polym 37:245
18. Löfas S, Johnsson B (1990) J Chem Soc Chem Commun 1526
19. Jönsson U, Fägerstam L, Ivarsson B, Johnsson B, Karlsson R, Lundh K, Löfas S, Persson B, Roos H, Ronnberg I, Sjölander S, Stenberg E, Stahlberg R, Urbaniczky C, Östlin H, Malmqvist M (1990) Biotechniques 11:620

20. Karlsson R, Michaelsson A, Mattsson L (1991) J Immunol Methods 145:229
21. Chaiken I, Rose S, Karlsson R (1992) Anal Chem 201:197
22. Malmqvist M (1993) Nature 361:186
23. Niikura K, Nagata K, Okahata Y (1996) Chem Lett 863
24. Bardea A, Dagan A, Ben-Dov I, Amit B, Willner I (1998) Chem Commun 839
25. Patolsky F, Lichtenstein A, Willner I (2000) J Am Chem Soc 122:418
26. Yamaguchi H, Oka F, Kamachi M, Harada A (1999) Kobunshi Ronbunsyu 56:660
27. Harada A, Yamaguchi H, Oka F, Kamachi M (1999) Proc SPIE Int Soc Opt Eng 3607:126
28. Yamaguchi H, Harada A (2002) Chem Lett 382
29. Yamaguchi H, Harada A (2002) Biomacromolecules 3:1163
30. Harada A, Okamoto K, Kamachi M, Honda T, Miwatani T (1990) Chem Lett 917
31. Harada A, Yamaguchi H, Tsubouchi K, Horita E (2003) Chem Lett 32:18
32. Harada A, Shiotsuki K, Fukushima H, Yamaguchi H, Kamachi M (1995) Inorg Chem 34:1070
33. Harada A, Fukushima H, Shiotsuki K, Yamaguchi H, Oka F, Kamachi M (1997) Inorg Chem 36:6099
34. Harada A, Yamaguchi H, Kamachi M (1997) Chem Lett 1141
35. Liou HH, Tsai MC, Chen CJ, Jeng JS, Chang YC, Chen SY, Chen RC (1997) Neurology 48: 1583
36. Brooks AI, Chadwick CA, Gelbard HA, Cory-Slechta DA, Federoff HJ (1999) Brain Res 823:1
37. Niewola Z, Hayward C, Symington BA, Robson RT (1985) Clinica Chimica Acta 148:149
38. Hogg PJ, Johnston SC, Bowles MR, Pond SM, Winzor DJ (1987) Molec Immun 24:797
39. Bowles MR, Pond SM (1990) Mol Immunol 27:847
40. Bowles MR, Hall DR, Pond SM, Winzor DJ (1997) Anal Biochem 244:133
41. Leatherbarrow RJ, Stedman M, Wells TNC (1991) J Mol Biol 221:361
42. Mazeran PE, Loubet JL, Martelet C, Theretz A (1995) Ultramicroscopy 60:33
43. Han W, Mou J, Sheng J, Yang J, Shao Z (1994) Biochemistry 34:8215
44. Zhang Y, Sheng S, Shao Z (1996) Biophys J 71:2168
45. Wang J, Jiang M, Nilsen TW, Getts RC (1998) J Am Chem Soc 120:8281
46. Esch MB, Baeumner AJ, Durst RA (2001) Anal Chem 73:3162
47. Shenton BW, Davis SA, Mann S (1999) Adv Mater 11:449