These values were within the frequently observed ranges for protein crystals. Structural refinements were carried out with the program *REFMAC* version 5.5.01[34] and *PHENIX* version 1.8.4.[35] The structures were visualized and manually modified with the *COOT* software.[36] The stereochemical configurations of the refined structures were validated with the program *PROCHECK*,[37] which showed that only 0.8% residues are in disallowed regions of a Ramachandran plot in the both crystal structures. The buried surface area was calculated with the program *PISA*[38] at the PDBe website. Interactions within the Fn3-Fab interface were assigned with the *CONTACT* program in the *CCP*4 suite or the *PISA* program. Data collection and refinement statistics are summarized in Table I.

MD simulations

Prior to MD simulations, the coordinates of the fragment of variable region of antibody (Fv) were extracted from the initial structure of the wild-type complex taken from the crystal structure in this work. We utilized *Discovery Studio* version 3.1 (Accelrys, San Diego, CA) to generate several missing residue coordinates (Fn3 residues from 31 to 35 and 83 to 85), and to computationally generate the mutant structure by replacing $Tyr^{L}50$ of the wild-type structure with Ala (LY50A). The MD simulations were performed for each of the wild-type Fv-Fn3 complex and LY50A Fv-Fn3 complex. The structure was energetically minimized after adding 20,547 water molecules, 63 Na^+ ions, and 60 Cl^- ions to reproduce the saline physiological environment. In this work, we used a modified version of the AMBER protein model,[39] and the TIP3P water model for describing the interaction of the solvated protein systems. Although the protein coordinates were restrained to those of the minimized structure, the water molecules and ions were equilibrated for 250 ps using a MD simulation. Subsequently, the positional restraints were removed, and three 400 ns MD trajectories were calculated with randomly generated initial velocities for each the wild type and LY50A complex, respectively. In all the simulations, the temperature and pressure were adjusted to 298 K and 1 atm with the Nose–Hoover thermostat and Berendsen barostat. For both the thermostat and the barostat, the relaxation time constants were set to 0.1 ps. To address the long-range coulombic interactions, the particle mesh Ewald method was used with a real space cutoff of 1 nm. The simulation time step was 2 fs. All chemical bond lengths were kept constant using the LINCS algorithm. Because the RMSD with respect to the crystal structure gradually increased for the first 100 ns, the latter 300 ns trajectories were used for the interaction energy analysis where the long-range electrostatic term was neglected. For systematic comparison, the same analyses were conducted for the hen egg lysozyme (HEL)-HyHEL10 complex (PDB ID code 2DQJ).[8] In this work, all MD trajectories were calculated using *GROMACS* version 4.5.5.[40]

ITC experiments

Prior to ITC experiments, wild type and LY50A mutant of B2212A scFvs and soluble ROBO1 (sROBO1) were prepared (See Supporting Information). Thermodynamic parameters of the interaction between B2212A scFv antibodies and its antigens were determined using MicroCal iTC200 system (GE Healthcare). The experimental conditions were as follows: in a calorimeter cell, the scFv fragments, at a concentration of 5–10 μM in phosphate-buffered saline [10 mM phosphate buffer (pH 7.4), 150 mM NaCl and 45 mM KCl], were titrated with 55.4–96.2 μM solution of antigens in the same buffer at 25°C. The antigen solution was injected 25 times. Thermograms were analyzed with *Origin* 7 software (GE Healthcare) after subtraction of the thermogram against a buffer background. The enthalpy change (ΔH) and binding constant (K_a) for the antigen–antibody interaction were directly obtained from the experimental titration curve fitted to a one-site binding isotherm. The dissociation constant (K_d) was calculated as $1/K_a$. The Gibbs free energy change ($\Delta G = -RT \ln K_a$) and the entropy change (ΔS $\Delta(-\Delta G + \Delta H)/T$) for the association were calculated from ΔH and K_a.

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Review

Generation of antibodies against membrane proteins ☆

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ABSTRACT

The monoclonal antibody has become an important therapeutic in the treatment of both hematological malignancies and solid tumors. The recent success of antibody-drug conjugates (ADCs) has broadened the extent of the potential target molecules in cancer immunotherapy. As a result, even molecules of low abundance have become targets for cytotoxic reagents.
The multi-pass membrane proteins are an emerging target for the next generation antibody therapeutics. One outstanding challenge is the difficulty in preparing a sufficient amount of these membrane proteins so as to be able to generate the functional antibody. We have pursued the expression of various membrane proteins on the baculovirus particle and the utilization of displayed protein for immunization. The strong antigenicity of the virus acts either as a friend or foe in the making of an efficient antibody against an immunologically tolerant antigen. This article is part of a Special Issue entitled: Recent advances in molecular engineering of antibody.

1. Introduction

Monoclonal antibodies (mABs) have attracted considerable interest in the treatment of cancer and autoimmune disorders [1–4].

In cancer, the therapeutic antibody targets are cell surface molecules which are predominantly comprised of membrane proteins. The main protein targets in cancer immunotherapy are growth factor receptors or overexpressed differentiation antigens. These target proteins are not strictly tumor specific, but perform an important function in the targeting cancer drugs. Furthermore, recent studies have demonstrated the heterogeneity of cancer cells and the problem of cancer stem cells acting in their own microenvironment [5,6]. To help overcome these difficulties, both toxins and radionuclide-conjugated antibodies have been developed for the purpose of improved cytotoxic activity [7–9]. Antibody engineering has also evolved so as to allow the design of bispecific antibodies, which increase the cytotoxic efficiency by either the conjugation of immunoadoptive target molecules or the blockade of immune checkpoints [10–12].

Thus, one of the important issues for the next generation of therapeutic antibodies is to obtain a higher affinity for the purpose of targeting less abundant surface molecules. Although it is difficult to raise high affinity antibodies against membrane proteins, there have been many useful strategies put forth for therapeutic antibodies, such as the use of DNA immunization [13] or phage-display [14]. Each of these technologies has its own merits and demerits, and the selection of which to use in order to obtain an effective antibody is largely dependent on the characteristics of the target protein. We here introduce our baculovirus display technology for generating mABs against membrane proteins.

2. Membrane protein preparation

The major target membrane proteins used for cancer immunotherapy thus far have been either cell surface receptors or adhesion molecules (Table 1). There is one anti-G protein-coupled receptor (GPCR) mAB on the market for the treatment of leukemia. The others include single-pass membrane receptors, and there is no ion channel or transporter protein in clinical use or phase III trials. The blockade of receptor function other than antibody-dependent cell-mediated cytotoxicity (ADCC) has certain favorable aspects that make it a good target choice. However, as the armed antibody therapeutics such as ADCs, radioimmunoconjugates and bispecific antibodies with an immunoadaptive recognition site have come to market, it has come to be expected that the less abundant proteins, such as GPCRs or other multi-pass membrane proteins, would eventually be realized as a target for cancer therapeutics.

In this regard, there have been difficulties in generating high affinity mABs against multi-pass membrane proteins. These antibodies are needed to recognize the native state of membrane proteins on the cell surface. The most pressing problems include (1) the difficulty of the preparation of a large amount of the protein in the proper conformation [15] and (2) the immunological tolerance that occurs due to the high level of sequence homology between species in the case of many critically important proteins.

☆ This article is part of a Special Issue entitled: Recent advances in molecular engineering of antibody.

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Table 1
Therapeutic target membrane proteins of monoclonal antibodies for cancer in Phase 3 trials or on the market.

Target classes	Target and type	Name of mAB	Indicated diseases
Receptor (GPCR, 7TM)	CCR4	Mogamulizumab	Adult T-cell leukemia/lymphoma
Receptors (non-GPCR)	CD30 (TNFRSF8)	Brentuximab vedotin	Hodgkin's lymphoma
	EGFR	Cetuximab	Colorectal cancer
	EGFR	Panitumumab	Colorectal cancer
	EGFR	Necitumumab	NSCL cancer
	EGFR	Nimotuzumab	SCC(head neck), glioblastoma multiforme
	EGFR	Zalutumumab	Head and neck cancer
	HER2	Pertuzumab	Metastatic breast cancer
	HER2	Trastuzumab	Breast cancer
	IGF-1R	Dalotuzumab	Metastatic colorectal cancer
	Folate receptor a	Farletuzumab	Ovarian cancer
	CD80 (ligand for CD28, CTLA-4)	Galiximab	NHL
	CD20	Ibritumomab	NHL
	CD20	Obinutuzumab	Diffuse large B cell lymphoma, CLL, NHL
	CD20	Ofatumumab	Diffuse large B cell lymphoma, CLL, NHL
	CD20	Rituximab	NHL
	CD20	Tositumomab	Malignant lymphoma, CLL
	CD22 (SIGLEC family)	Inotuzumab ozogamicin	ALL, NHL
	CD22	Moxetumomab pasudotox	Hairy cell leukemia
	CD33 (SIGLEC family)	Gemtuzumab ozogamicin	Acute myeloid leukemia
	VEGFR2	Ramucirumab	Metastatic gastric or gastroesophageal junction adenocarcinoma; breast cancer; hepatocellular carcinoma
	CD4	Zanolimumab	Cutaneous T-cell lymphoma
	CD2	Elotuzumab	Multiple myeloma
	cMET (HGFR)	Onartuzumab	NSCL cancer, gastric cancer
	PD1	Nivolumab	NSCL cancer, renal cell carcinoma, melanoma
	CTLA-4	Ipilimumab	Advanced melanoma, sepsis
	CTLA-4	Tremelimumab	Metastatic melanoma
Adhesion molecule	EpCAM/CD3	Catumaxomab	Malignant ascites
Enzyme	Carbonic anhydrase ix (metalloenzyme)	Girentuximab	Non-metastatic renal cell carcinoma
Others (oncofetal or differentiation antigen)	CD52 (GPI anchor)	Alemtuzumab	B-cell chronic lymphocytic leukemia, GVH(graft versus host), Multiple Sclerosis
	5 T4	Naptumomab estafenatox	Advanced renal cell carcinoma

Based on the data from ref. [2]; mAB, monoclonal antibody; GPCR, G protein coupled receptor; TM, trans-membrane; CCR4, C-C chemokine receptor type 4; CD, cluster of differentiation; TNFRSF8, tumor necrosis factor receptor superfamily, member 8; EGFR, epidermal growth factor receptor; NSCL, non-small cell lung ; SCC, squamous cell carcinoma; HER2, human epidermal growth factor receptor 2; IGF-1R, insulin-like growth factor-1; CTLA-4, cytotoxic T-lymphocyte antigen 4; NHL, non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia; VEGFR2, vascular endothelial growth factor receptor 2; cMET, met proto-oncogene; HGFR, hepatocyte growth factor receptor; PD-1, programmed death-1; EpCAM, epithelial cell adhesion molecule

The use of a peptide fragment or small domain as an immunogen is frequently unsuccessful in the effort to obtain specific antibodies which recognize cell surface antigens. This is due to the difference in conformation between the peptide and the protein. For single-pass membrane proteins, the use of entire extracellular portion of these proteins is reportedly largely successful. For example, the use of the Fc fusion protein [16] has been shown to be largely successful in the preparation of the large amount of protein necessary for immunization.

In recent years, the preparation has been sufficiently improved that several multi-pass membrane proteins, including GPCRs, have been obtained in an amount that allows crystallography [17,18]. The solubilized proteins are reconstituted in phospholipid vesicles in which the adjuvant molecule is also incorporated for immunization [19,20].This method is very useful in the generation of mABs for crystallization probe [21,22]. As the proteins are incorporated in a random orientation in the liposome, the antibodies generated by this method tend to recognize the cytosolic side of the membrane protein, probably due to the immunological tolerance of the exposed side.

We observed that a relatively large amount of membrane proteins are displayed on the budded baculovirus (BV) particles during the expression of membrane proteins of endoplasmic reticulum (ER) origin [23]. Upon further investigation we found this BV display useful for the generation of antibodies against the multi-pass membrane proteins that are typically difficult to obtain in sufficient amounts. In addition to the whole protein display method, there is also a BV display technique using a fragment peptide as a fusion protein with the viral membrane protein gp64 [24,25] (Fig. 1).

3. Baculovirus display of membrane proteins

3.1. Whole protein

The expression of functional membrane proteins on BV particles was first reported by Loisel et al. [26,27]. They attempted to recover the β2-adrenergic receptor (β2-AR) on the virus-like particles (Gag-particles) from Spodoptera frugiperda (Sf9) cells by infecting recombinant baculovirus harboring the human immunodeficiency virus type 1 (HIV-1) Pr55Gag protein gene. Contrary to their expectations, the β2-AR did not appear on the gag-particles, but on the BV particles. The receptor expressed on the virus was functionally coupled to both the Gs and adenylyl cyclase of host insect cell origin, and exhibited a higher level of activity than that recovered from the Sf9 cell membrane fraction. The membrane protein collected from the Sf9 cells often includes a substantial proportion of inactive protein that is difficult to separate and a major cause of the difficulty in raising the antibody. We encountered this phenomenon when expressing a membrane protein of endoplasmic reticulum origin [23]. We then examined GPCR expression on the BV using the leukotriene B4 (LTB4) receptor (BLT1) [28]. In this case, BLT1 couples to the Gi isoform of a trimeric G-protein which inhibits adenylyl cyclase. As the Gi isoform is not expressed in Sf9 cells, we were able to recover the highly sensitive LTB4 binding activity on the BV after the co-infection of recombinant baculoviruses which harbor trimeric Gi-protein subunit genes [28]. These show that BV has the capacity to display not only a single membrane protein by itself, but also the reconstituted functional protein complex. On this point, we have further demonstrated that the effector protein adenylyl cyclase,

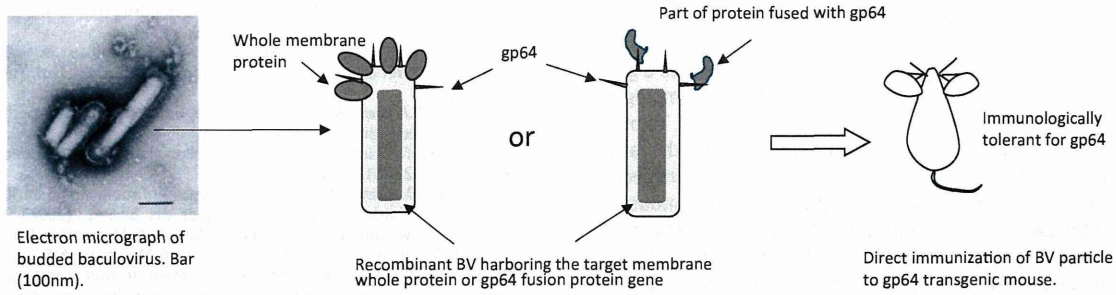

Fig. 1. The use of baculovirus display of membrane protein for immunization.

a multi-pass membrane protein, which was also expressed at the same time as the whole signaling complex, was reconstituted on BV [29].

One more point to be noted on the BV display of the GPCRs is that even the olfactory receptors, which are commonly considered to be the most difficult GPCR type in terms of cell surface expression, are displayed on BV in the form of the functional complex [30]. We have thus far confirmed a couple of dozen GPCRs with the BV display, although the expression level differs by orders of magnitude between them. The reason for the different expression levels is not known at present, but may be related to the difference in the expression patterns in the Sf9 host cell membrane.

The mechanism of the BV ER protein display is unknown. However, an assembly of viral particles around the nuclear envelope has been observed [26]. Considering the functional equivalency of the nuclear envelope and rough ER, it is speculated that the BV envelope may contain some portion of the ER before budding from the plasma membrane. We have checked the expression of several of the known ER proteins, such as sterol responsive element binding protein (SREBP) or SREBP cleavage activating protein (SCAP) on BV [23]. 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in its full length form was also recovered in the BV fraction and possessed enzymatic activity (Unpublished data). We have also demonstrated multi-pass membrane proteins other than receptors, such as a transporter (pepT1 [31]), a channel (aquaporin 4 [32]), adhesion molecules (claudins [33]) and certain enzymes (DHHC palmytoilation enzymes [34] and γ-secretase complex [35]). All of the membrane proteins tested so far, with a few exceptions, such as vesicle transfer proteins, have been functionally displayed on the viral particles. Such functional expression of membrane proteins is highly useful in the construction of various assay systems [36–39].

3.2. Domain fusion protein

The display of foreign protein fused with the baculoviral membrane protein gp64 was reported by Boublik et al. [24]. The use of the gp64-fusion protein for immunization is useful for proteins such as the zinc finger family of proteins, in which the similar structure of the family members makes it difficult to obtain a specific antibody due to the sequence similarity of the zinc finger domain. Lindley et al. reported efficient immunization using a fusion of the Liver X Receptor α (L X Rα) with gp64 [40]. As gp64 is a unique membrane protein of baculovirus origin and is necessary for infection, the expression of the fusion protein is confined to the lower level than gp64 itself. In our experience, the use of 30 to 50 amino-acid residues is most efficient for the stable expression of the fusion protein. Thus, the gp64-fusion is useful for generating an antibody to partial domains of the long external portion of the membrane protein [41].

4. Immunization of the displayed membrane protein

Once the recombinant virus harboring the target membrane protein gene is established, it is relatively easy to obtain a sufficient amount of antigen by infecting recombinant BV in Sf9 cells. Since certain proteins are very susceptible to degradation, there are several points to keep in mind when preparing the BV with the immunizing antigen. It is recommended the BV sample be prepared just before the injection, or the addition of 1 mM ethylenediaminetetraacetic acid (EDTA) and 50 μM E-64 is helpful for the purpose of preservation for one to two months. The storage of BV is carried out at 4 °C, since the freeze thaw process induces aggregation. The BV envelope is so fragile that excessive pipetting must be avoided. We used overnight standing still dispersion of pelleted BV in phosphate buffered saline (PBS) at 4 °C. We usually use *pertussis* toxin for the adjuvant because other lipid-base adjuvants may cause either the destruction of the envelope or the unwanted solubilization of the displayed membrane protein [42].

The most serious problem with BV immunization is its own strong antigenicity. The precise mechanism is still unclear, but it is well known that BV evokes a strong immune reaction and thus is a good adjuvant [43–46]. In case of whole membrane protein display, especially GPCRs, the amount of the displayed target protein is far less than that of gp64. We found so many clones reacted with gp64 that it was difficult to get the reactive clones to the target protein.

A transgenic mouse harboring the gp64 gene was therefore generated. The gp64-transgenic mice exhibited the expression of a large amount of the gp64 protein and a tolerance to gp64 [42]. The hybridoma clones obtained from the gp64-transgenic mice displayed considerably reduced reactivity to gp64.

MABs with ADCC and complement-dependent cytotoxicity (CDC) activities against peptide transporter 1 (PepT1)-expressing pancreatic tumor cells have been generated using this technique [42].

5. Immunization of single-pass membrane proteins

The immunization method of the BV-displayed protein is applicable to the single-pass membrane proteins. As the single-pass membrane proteins usually have a long extracellular portion readily prepared in large amounts, there is generally no serious problem in generating a functional antibody. However, in the case of ROBO1, which is an axonal guidance receptor of single-pass membrane protein with five immunoglobulin-like domains and three fibronectin-like domains in its extracellular portion, we have obtained reactive antibodies to ROBO1 expressing cells by means of BV-displayed whole protein [47]. ROBO 1 is a surface antigen over-expressed on liver cancer cells and is a good candidate for immunotherapy [48]. The monoclonal antibodies generated with BV immunization belong mostly to the IgG2 subclass, so the ADCC activity is relatively weak. However, the radioisotope labelled form exhibited potent anti-tumor activity [47].

We also were able to obtain several neutralizing monoclonal antibodies against γ-secretase by the immunization of BV expressing human nicastrin [49,50]. The antibodies we obtained recognized human nicastrin very well but not murine nicastrin at all. It turned out their epitopes resided around the glycosylation site where the amino acid sequence is different between two species [49]. This suggests immunological tolerance to self-antigen.

6. The use of a knock-out mouse for the target gene

The generation of the reacting antibody to GPCRs provides a good example of immunological tolerance since they are highly homologous across species in addition to having a low level of expression. To overcome this problem, we employed a knockout mouse lacking the target protein gene for immunization [42]. We crossed a C-C chemokine receptor type 2 (CCR2) knock-out mouse and gp64 transgenic mouse to generate a CCR2 negative and gp64 positive mouse. We have obtained several monoclonal clones reactive to CCR2-expressing Chinese hamster ovary (CHO) cells. The application of a knock-out mouse is useful for the target proteins having a high homology across species. For immunization, a Balb/c background is preferred so that the preparation of the knock-out mouse for immunization sometimes requires a burdensome task. Furthermore, a biologically important molecule tends to result in embryonic lethality when the gene is deleted.

Thus, we have developed alternative immunization method for autologous antigens by preparing a nude mouse transfused with T-regulatory lymphocytes (Treg)-depleted spleen cells. This mouse provided reactive antibodies to mouse protein effectively [51]. This technique, however, turned out to be not totally compatible with the BV display, since the Treg depletion from gp64 transgenic mouse spleen cells restored the reactivity to gp64 in the transfused mice (unpublished observation).

7. Future perspectives

It has proven difficult to use a BV display for the kinetic study of antigen-antibody interactions. This is because the virus particle is relatively large, as indicated in Fig. 1, so that the BV-displayed membrane protein is beyond the limit of the evanescent field of Surface Plasmon Resonance measurement. Recently, we found that the Kinetic Exclusion Assay (KinExA) is applicable to this purpose (manuscript in preparation). The kinetics of KinExA provides a means to evaluate the affinity of an antibody and the specific amount of antigen [52]. This expands the potential for generating high affinity therapeutic mABs against additional targets, such as viral antigens. However, the amount of the membrane proteins displayed on BV is not always enough for immunization, especially in the case of some GPCRs. The use of a gp64 null virus [53], perhaps in combination with other techniques to increase the antigen concentration [39], will be investigated for the purpose of achieving more efficient immunization.

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A new practical technique for high quality protein crystallization with the solution stirring technique at the interface between high-concentrated hydrogel and solution

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A new practical technique for high quality protein crystallization with the solution stirring technique at the interface between high-concentrated hydrogel and solution

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We developed a new crystallization technique combining the solution stirring technique and crystallization at the interface between high-concentrated hydrogel and solution (the on-gel stirring technique). The solution stirring effect on the crystal quality of insulin was evaluated by an average difference between temperature factors determined by the slopes of the relative Wilson Plot. Higher-quality insulin crystals could be obtained under the stirred environment. The environmental stability of insulin crystals grown at the interface between hydrogel and solution for temperature change was tested. The dissolution point of a gel-interface crystal was 2 K higher than that of a solution-grown crystal. The quality of an insulin crystal grown by the on-gel stirring technique was finally evaluated and it was better than the solution-grown crystals with/without stirring and the gel-interface grown crystals. These results confirm that the on-gel stirring technique is a practical way to obtain high-quality crystals with improved environmental stability.

1. Introduction

Three-dimensional (3D) structures of proteins are utilized in the understanding of protein functions and interactions, as well as in the development of new pharmaceutical products. The 3D structures of proteins are often determined by X-ray structural analysis of a single protein crystal. However, protein crystals obtained using conventional crystallization methods are often very small, low quality and weak. Because of their fragility, they get damaged easily in the handling process for X-ray diffraction data collection. Such the damage further reduces the original quality of the crystals, and these crystals are then unsuitable for high-quality X-ray data collection. Therefore, there is a clear need to develop new methods for obtaining large, high-quality crystals with improved environmental stability.

To date, crystallographers have developed many protein crystallization techniques, such as crystallization in microgravity,[1,2] in magnetic fields,[3–7] and in a hydrogel concentrated up to 0.6% w/v.[8–10] We previously developed a crystallization technique with solution stirring. Comparing these techniques with crystal growth under normal convection on the ground, the desire to create a uniform growth environment was found to be one of a common concern. Our group has succeeded in producing high-quality crystals using both the stirring technique[11] and a high-concentrated hydrogel.[12–15] We also demonstrated that crystallization with highly concentrated (~2% w/v) hydrogels promotes crystal nucleation[16] and produces crystals that can adapt to environmental changes (temperature change, osmotic shock, etc.).[12,17]

To obtain protein crystals with higher quality and improved environmental stability, we suggest a new technique combining these two techniques, i.e., crystallization at the interface between a high-concentrated hydrogel and a stirred solution. Crystallization using a hydrogel has two significant effects on crystal growth. The first is the provision of a diffusion controlled environment, and the second is the enhancement of crystals tolerances toward environmental change by gel fiber irruption into the crystals. We focused our attention on the latter issue, and combined the technique with crystallization using a stirred solution, i.e., the on-gel stirring technique (Fig. 1). When nucleation occurs on the hydrogel, the crystal grows at the interface between the hydrogel and the solution. In such cases, the solution-stirring technique may be advantageous to keep the surfaces of the crystals constantly exposed to the solution.

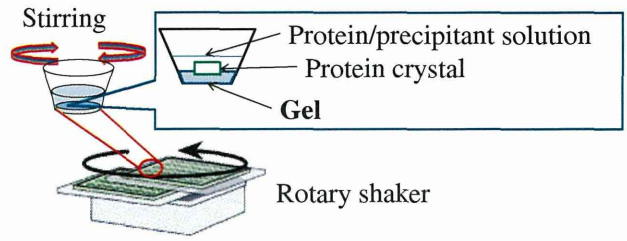

Fig. 1. (Color online) Schematic illustration of the crystallization setup for the on-gel stirring technique.

In this work, the solution-stirring effect on the crystal quality at several stirring speeds was investigated to determine the appropriate solution stirring speed for the growth of high-quality crystals. The environmental stability of crystals grown at the interface between the gel and the solution was then estimated. At the end of this work, we applied the on-gel stirring technique on insulin crystal and succeeded to improve the temperature factor. These results will confirm the success of this new technique, the on-gel stirring technique.

2. Experiment

2.1 Crystallization

Insulin from bovine pancreas was purchased from Sigma-

