ADVANCED NANOBIOMEDICAL APPLICATION OF THE PHOSPHORYLCHOLINE-POLYMER SURFACE TECHNOLOGY (PCST)

Kazuhiko Ishihara[1], Madoka Takai[1], and Junji Watanabe[2]
[1]Department of Materials Engineering and Center for NanoBio Integration
The University of Tokyo, Tokyo, 113-8656, Japan
[2]Department of Applied Chemistry
Osaka University, Osaka 565-0871, Japan

Introduction

How to make an excellent biointerfaces between biological system and artificial materials is interesting and important issue in biomedical, bioengineering, and bioscience research fields. Particularly, recent progress in the biotechnology requires much more high performance on the biointerfaces. In this communication, It is presented a special concept for preparing biointerfaces based on "***PC surface technology (PCST)***", which are phospholipids and phospholipid polymers assembling technology. By using this PCST, the surface of the artificial materials converts to "***artificial cell membrane***" surface[1]. That means, this technology is new and key bridge to connect biological field and materials engineering.

Recently, our interests in biomaterials shifted to nanobiomaterials, which is integrated with nanofabrication. Nano-scaled fabrication is based on molecular assemblies such as micelles, self-assembled monolayers, and supramolecular compounds. Also, many kinds of nano-scaled devices such as bio-chips and nanoparticles have been proposed and designed. Nano-scaled chemistry and nanofabrication are focused on chemical reactions and sensing. The most fascinating phenomena, chemical reactions, are created in a nano-scaled world, but never carried out on an ordinary scale. In these nano-scaled devices, the biointerface property is a dominant factor in order to provide excellent performance. To enhance biofunctionalization it is of at most importance for the nano-scaled device to suppress non-specific protein adsorption since the surface is exposed to many biological components; blood, serum, and cells. Only a few researchers have understood the importance of materials in the design of biointerfaces and bioconjugations in the nanoscale [2-4]. Here, newly engineered materials using bioinspired phospholipid polymer chemistry for biofunctionalization have been summarized particularly with focus on the phospholipids polymer nanoparticles for molecular diagnosis. The phospholipid polymers can be available in numerous situations for specific biomaterials, and could function as molecular machine [5]. Ishihara *et al*. proposed a fundamental concept for the synthesis of phospholipid polymers in the 1990's [6,7]. Now, we easily purchase many kinds of products containing the phospholipid polymer; cosmetics, eye care products, textile goods, and fine chemicals for advanced bioreactions, which were designed through collaborations between Dr. Ishihara and many companies worldwide. This is a typical process and development from the laboratory materials to commercial products. The developed phospholipid polymer synthesis has been recognized worldwide, and many researches were subsequently reported (see [1] for an excellent review). Further research in

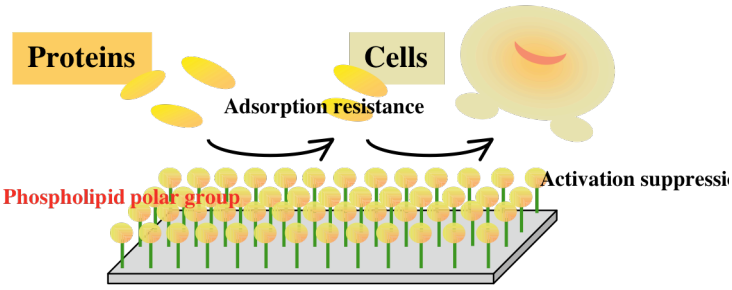

Fig. 1. Concept of PCST for preventing bioreactions at the surface of devices

nanofabrication will provide us better "Quality of Life", especially when biomaterials are designed based on the phospholipid polymers.

Nanofabrication for Biomedical Applications

Nanoparticles are one good substrate for bioconjugation. They have (i) relatively high specific surface area, (ii) good dispersivity in aqueous media, and (iii) can be combined with nano-processed biochips. These characteristics can be used in a wide variety of applications, for example, medical diagnosis, drug delivery carriers for targeting, micro-total analysis systems, and biosensors. The most important factor in improving the nanoparticles is the enhancement of specificity to the target biomolecule such as antigen, substrate, and DNA (these are selectivity); and suppression of non-specific interaction (sensitivity). Thus, bioinspired phospholipid polymers are key materials in the research on and development of nanoparticles.

Key Materials for Nanofabrication

We have recently proposed the assembly of phospholipids polar group on the nanoparticles. The nanoparticles are generally unstable as colloid particles in aqueous media. A hydrophilic moiety and ionic groups are incorporated onto the surface to prepare the interface. One of the anomalous interfaces is the cell membrane, which is composed of phospholipid molecules, glycoproteins, and channel-forming proteins, to provide not only dividing between the cytoplasm and the outer environment but also to communicate via antenna molecules and channels. Ishihara et al. designed and synthesized a novel functional monomer with a phospholipid polar group, 2-methacryloyloxyethyl phosphorylcholine (MPC), for fabrication of the cell membrane structure as biointerface [1,8,9]. The MPC (methacrylate derivative) easily polymerized with any kind of acrylate and methacrylate monomers by using conventional radical polymerization, living radical polymerization, and atom transfer radical polymerization [8,9]. Finely designed polymers with phospholipid polar groups were easily prepared by arrangement of the polymerization techniques. The phospholipid polymer could form the cell membrane-like interface using coating, polymer blending, and polymer graft techniques. A typical phospholipid polymer is copolymerized MPC with alkyl methacrylate such as *n*-butyl methacrylate (BMA) and dodecyl methacrylate. The phospholipid polymer provided a very bio-inert interface, that is, non-specific interactive biointerface on the diversity materials were obtained and many biomedical devices were developed using the PCST [10,11]. The stability of the phospholipid polymer-modified enzyme was also significantly prolonged in comparison with the native enzyme [5]. Furthermore, the phospholipid polymer spontaneously formed nano-structured aggregations, indicating amphiphilic and surfactant-like properties [12]. The employment of a polymer based biomimetic surface is a promising approach to prepare nano-scaled devices for biofunctionalization

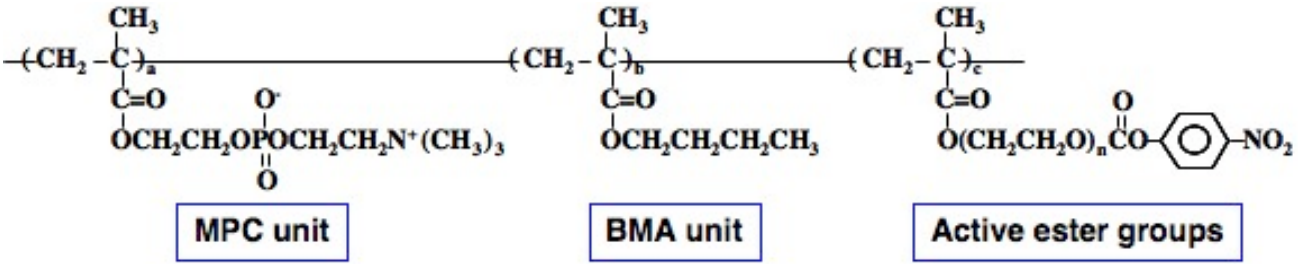

Fig. 2. Chemical structure of PMBN for preparation of bioconjugate nanoparticles

The phospholipid polymer was designed for the bioconjugation with biomolecules such as proteins, peptide, and DNA. We synthesized a functional monomer, that is, a *p*-nitrophenyloxycarbonyl poly(oxyethylene) methacrylate (MEONP) having an active ester linkage for bioconjugation[13,14]. The MPC, MEONP, n-butyl methacrylate (BMA) were copolymerized to prepare the bioconjugate phospholipid polymer (PMBN; Fig. 2), by a conventional radical polymerization technique with 2,2'-azobisisobutyronitrile as an initiator. This polymer involves dual functions; suppression of non-specific adsorption from our living body and connection of biomolecules via active ester group. The bioconjugate phospholipid polymer having 40 mol% of MPC could easily dissolve in water, and 5-8 mol% of active ester groups were incorporated.

Bioconjugate Nanoparticles

Bioconjugate nanoparticles were prepared by the solvent evaporation methods under the systematic design of core materials; polystyrene (PS) as a conventional polymer and poly(L-lactic acid) (PLA) as a biodegradable polymer. According to the molecular design of the bioconjugate phospholipid polymer, the hydrophobic chains, n-butyl group, were considered to penetrate on the surface of nanoparticles. The phospholipid polar groups and active ester groups, which formed domains, were concentrated at the nanoparticle surface in order to stabilization of the interface, and the *p*-nitrophenyl ester groups can freely conjugate with biomolecules. In this section, we investigated whether the characteristics of the nanoparticles would alter by changing the core materials. As core polymer materials, PLA and PS were used to prepare PMBN/PLA or PMBN/PS nanoparticles by a solvent evaporation technique in aqueous medium [13-16]. In this process, the PMBN was utilized as an emulsifier, and the polymer concentration regulated the average diameter of the nanoparticles. The diameter decreased with increasing the initial concentration of PMBN.

Biofunction on Nanoparticles

One biofunctional enzyme reaction was evaluated on the phospholipid polymer nanoparticles. Particularly, a sequential enzymatic reaction was designed. The most favorable characteristic is the

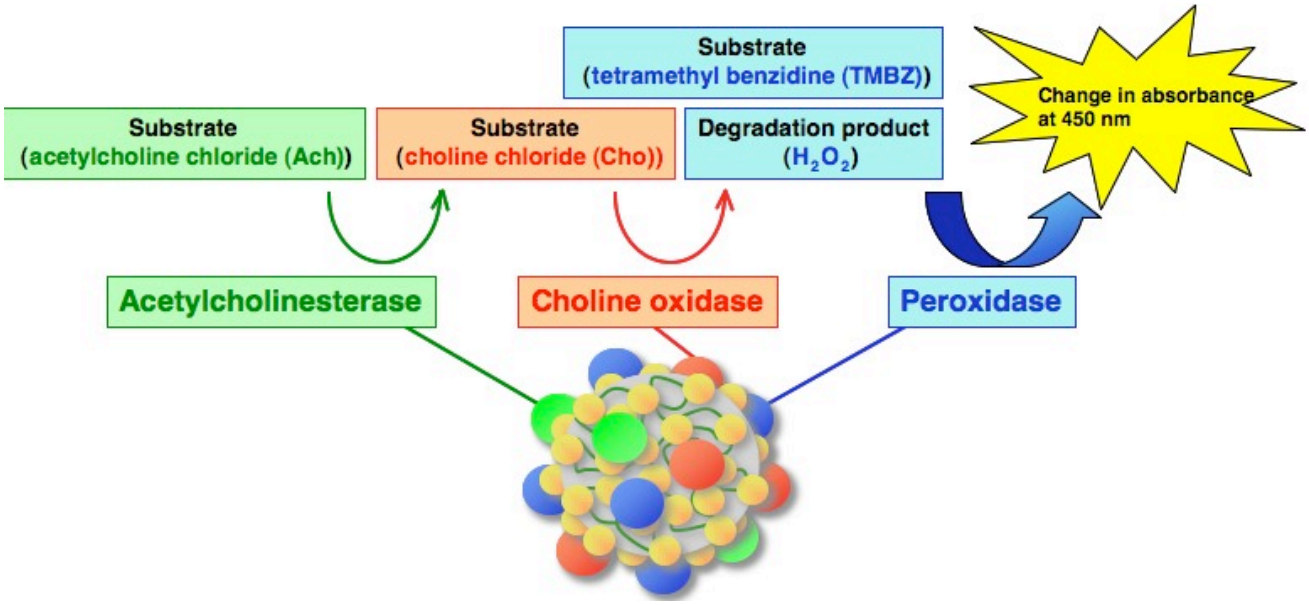

Fig. 3. Sequential enzymatic reaction with enzymes immobilized on the nanoparticles covered with artificial cell membrane.

local concentration of enzyme on the nanoparticles. To increase local concentration is effective for valuable and rare biomolecules.

We have recently proposed a novel signal amplified diagnosis system using sequential enzymatic reaction on nanoparticles (Fig. 3). The nanoparticles are composed of the phospholipid polymer with active ester groups and polystyrene core. The amplified signal was evaluated by using choline oxidase and peroxidase, which were co-immobilized onto the nanoparticles. The choline oxidase reacts with choline chloride, and hydrogen peroxide is produced. The produced hydrogen peroxide is used as a substrate in the next enzymatic reaction by peroxidase. In this sequential reaction, the amount of tetramethyl bendizine will be evaluated by change in absorbance; by single enzymatic reaction (peroxidase) and/or by sequential enzymatic reaction (choline oxidase and peroxidase).

The active ester group was labile to the primary amino group, and a carbamate linkage was produced for bioconjugation. We have already estimated the conversion of the active ester linkage on the nanoparticles, and 40% of the active ester linkage was converted by a reaction with proteins [16]. Two kinds of enzymes, choline oxidase and peroxidase, were co-immobilized onto the surface, and the sequential enzymatic reaction was then evaluated. The combination of the enzymes displayed their communication via the degradation product (hydrogen peroxide(H_2O_2). As a substrate, choline chloride (Cho), tetramethyl benzidine (TMBZ), and H_2O_2 were used. The Cho was oxidized by the choline oxidase, and H_2O_2 was newly produced as a degradation product. The produced H_2O_2 was used for the next enzymatic reaction; the oxidation of TMBZ by peroxidase. The H_2O_2, which was originally added to the media, could also be used as substrate.

Two kinds of protocol regarding the enzymatic reaction were examined; (i) TMBZ and H_2O_2 were added to the suspension and (ii) Cho was added to the suspension with TMBZ and H_2O_2 (TMBZ/Cho). In the case of protocol (i), only the enzymatic activity of the peroxidase was evaluated. On the other hand, newly produced H_2O_2 would be an enhancer for the sequential enzymatic reaction (protocol (ii)). The result of the enzymatic reaction was evaluated by the change in absorbance at 450 nm . The enzymatic reaction on the nanoparticles was significantly greater than that of a simple enzyme solution, when TMBZ was added as the substrate as shown in Fig. 4. The total amount of enzymes in the enzyme solution was larger than that of the immobilized enzyme on the nanoparticles, because the concentration of the enzymes in the solution was the same as the feed concentration for the preparation of the enzyme-immobilized nanoparticles. Taking the total amount of the enzyme concentration into account, it is considered that choline oxidase and peroxidase were locally concentrated on the particle surface in comparison with the solution. Furthermore, the sequential enzymatic reaction was compared. The change in absorbance increased with the addition of TMBZ/Cho, which was twice as large than that of the addition of only TMBZ. It was considered that the increased enzymatic activity was based on choline oxidase, and the produced H_2O_2 was effectively free to move to its binding site at the substrate. Initially, sufficient $H_2O_2$was added to the media to promote the enzymatic reaction; therefore, the increased enzymatic reaction was caused by the H_2O_2 newly produced by choline oxidase. In the

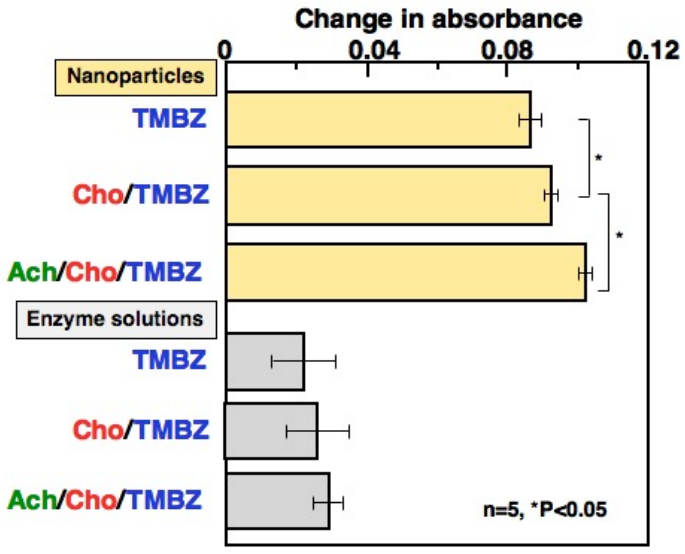

Fig. 4. Enhanced enzymatic activity of sequential reactions by immobilization on nanoparticles.

case of nanoparticles, the enzymes, choline oxidase and peroxidase, were closely immobilized onto the nanoparticles; therefore the diffusion pathway of the produced H_2O_2 was significantly shorter than that of the solution. Thus, the produced H_2O_2 was different from the originally added H_2O_2, which needed a long pathway to react with the peroxidase. On the other hand, no significant difference in the enzyme solution was observed between the single reaction (TMBZ) and the sequential reaction (TMBZ/Cho). This result indicated that the reaction with peroxidase proceeded through a single reaction, even if Cho was added to the media.

Conclusions

Nanofabrication is a promising technique that opens the door to a new scientific field, which integrates biochemistry, bioscience, material science, polymer chemistry, and nano-scaled processing. Under the nano-scale environment, surface properties (biointerface) are dominant factors, and thereby regulate biofunctions. Particularly, non-specific protein adsorption is a typical phenomenon on the nanofabrication. A series of phospholipid polymers are good candidates on nanofabrication for biofunctionalization.

References

[1] Watanabe, J., and Ishihara, K., "Biointerface, bioconjugation, and biomatrix based on bioinspired phospholipid polymers"., *In Handbook of Nanostructured Biomaterials and Their Applications*, Ed. by H. S. Nalwa, American Scientific Publishers, California, pp129 (2005).

[2] Siegers, C., Biesalski, M., and Haag, R., "Self-assembled monolayers of dendritic polyglycerol derivatives on gold that resist the adsorption of proteins", *Chem. Eur. J.*, **10**, 2831, 2004

[3] Jenkins, S. H., Heineman, W. R., and Halsall, H. B., "Extending the detection limit of solid-phase electrochemical enzyme immunoassay to the attomole level., *Anal. Biochem*., **168,** 292, 1988

[4] Ishihara, K., "Bioinspired phospholipid polymer biomaterials for making high perfomance artificial organs", *Sci. Tech. Adv. Mater.* **1**, 131, 2000

[5] Miyamoto, D., Watanabe, J., and Ishihara, K., "Effect of water-soluble phospholipid polymers conjugated with papain on the enzymatic stability", *Biomaterials*, **25**, 71, 2004

[6] Ishihara, K., Ueda, T., and Nakabayashi, N., "Preparation of phospholipid polymers and their properties as hydrogel membrane", *Polym. J.*, **23**, 355,1990

[7] Ishihara, K., Oshida, H., Endo, Y., Ueda, T., Watanabe, A., and Nakabayashi, N., "Hemoconpatibility of human whole blood on polymers with a phospholipid polar group and its mechanism", *J. Biomed. Mater. Res*., **26**, 1543,1992

[8] Inoue, Y., Watanabe, J., Takai, M., and Ishihara, K., "Surface characteristics of block-type copolymer composed of semi-fluorinated and phospholipid segments synthesized by living radical polymerization"*, J. Biomater. Sci. Polym. Edn*., **15**, 1153, 2004

[9 Inoue, Y., Watanabe, J., Takai, M., S.Yusa, and Ishihara, K., "Synthesis of sequence-controlled copolymers from extremely polar and apolar monomers by living radical polymerization and their phase-separated structures"*, J. Polym. Sci. Part A: Polym. Chem*., **43**, 6073, 2004

[10] Moro, T., Takatori, Y., Ishihara, K., Konno, T., Takigawa, Y., Matsushita, T., Chung, U.-I., Nakamura, K., and Kawaguchi, H., "Surface grafting of artificial joints with a biocompatible polymer for preventing periprosthetic osteolysis", *Nature Materials*, **3**, 829, 2004

[11] Yoneyama, T., Sugihara, K.-I., Ishihara, K., Iwasaki, Y., and Nakabayashi, N., "The vascular prosthesis without pseudointima prepared by antithrombogenic phospholipid polymer", *Biomaterials*, **23**, 1455, 2002

[12] Ishihara, K., Iwasaki, Y., and Nakabayashi, N., "Polymeric lipid nanosphere constituted of poly(2-methacryloyloxyethyl phosphorylcholine-co-n-butyl methacrylate)", *Polym. J.*, **31**, 1231, 1999
[13] Konno, T., Watanabe, J., and Ishihara, K., "Conjugation of enzymes on polymer nanoparticles covered with phosphorylcholine groups", *Biomacromolecule*s, **5**, 342, 2004
[14] Takei, K., Konno, T., Watanabe, J., and Ishihara, K., "Regulation of enzyme-substrate complexation by phospholipid polymer conjugates for cell engineering", *Biomacromolecules*, **5**, 858, 2004.
[15] Park, J.-W., Kurosawa, S., Watanabe, J., and Ishihara, K., "Evaluation of novel 2-methacryloyloxyethyl phosphorylcholine (MPC) polymeric nanoparticles for immunoassay of C-reactive protein detection", *Anal. Chem*., **76**, 2649, 2004
[16] Watanabe, J., and Ishihara, K., "Sequential enzymatic reaction and stability of biomolecules immobilized onto phospholipid polymer nanoparticles"., *Biomacromolecules,* **7**, 171, 2006