Development of

PEI-CEIA System for LPS Detection and

Cross-linked PEI Beads for Selective Removal of

LPS and DNA

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Contents

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Prologue

Recently, we are facing more problems of contamination in our foods, medicines and environments causing us sickness and in worst case death. The contaminants are organic, inorganic materials and microorganisms which levels should be monitored especially for the quality and safety of foods and environment because we are living in the age of mass production. Among them, such infections by bacteria such as *Salmonella* or *E. coli* O157 H7 became quite frequent. The accurate, quick, easy, safe and economical method to detect the lipopolysaccharide (LPS) of these Gram-negative bacteria cell wall as the antigen is our focus to develop an effective enzyme immunoassay method using poly(ethyleneimine)-cloth, (PEI-cloth). Here, the complete indispensable assemblies for the PEI-CEIA such as the substrate and stabilizer-blocker-diluent for the antibody-enzyme conjugate were newly developed. Also, LPS and recombinant DNA in preparations of vaccine or blood medical products which goes into our blood are considered as the harmful contaminants in medicines, and have to be removed. PEI in beads form found to be a capable specific remover of those harmful compounds from the biologically active solution and fluids.

In **Chapter One**, the general knowledge related to author's work is mentioned. Among methods to study and detect LPS, the enzyme immunoassay (EIA) is a well established method for quantitative way by measuring absorbency assayed in microwell or qualitative way by dot blot for visual identification on nitrocellulose or PVDF (polyvinylidene fluoride) membrane. A newly developed method is utilizing polymyxin B adsorbed on polyester cloth which can preferentially adsorb LPS in a sample mixture, and the captured LPS is further detected by EIA. The polymyxin-cloth EIA (p-CEIA) can be used for quantitative and qualitative ways. However p-CEIA had some short comings and the development of poly(ethyleneimine)-CEIA (PEI-CEIA) overcame these

problems. The backgrounds for the utilization of selective adsorptions of LPS and DNA by PEI and other polyamine spherical beads are discussed.

In **Chapter Two**, the preparation of 3,3',5,5' tetramethylbentizine (TMB) reagents and the blocker-stabilizer-diluent fraction are described. The design of new formulation of enzyme substrate TMB for enzyme–antibody conjugates and new preparation of a blocker which works as the stabilizer and diluent for the conjugate are presented. TMB is a substrate of the most commonly utilized marker enzyme, horse-radish peroxidase (HRP). This substrate is insoluble in water but the newly developed water solubilizing method made possible to prepare in a form of powder TMB reagents. In EIA, the blocker has very important function to prevent the nonspecific adsorption of enzyme-antibody conjugate and enhance sensitivity. It is crucial as well to stabilize the enzyme-antibody conjugate during assay. We have discovered a fraction isolated from powder skim milk solution that has unique characters work as a blocker and stabilizer and diluent.

Hiroshi Yamazaki, Kouchi Matsumoto
"Water soluble powdered formulation of reagent mixture containing water-insoluble reagents, and process for their production", US Patent, 5,910,423, June 8 (1999)

In **Chapter Three**, the preparation of a new type of EIA support using polyester cloth as the matrix for CEIA, which are polymyxin-cloth (p-cloth) and PEI-cloth, is described. The preparation of p-cloth, its improved form and the quality control monitor method are mentioned. It was developed two methods to prepare PEI-cloth through physisorption and chemisorption ways. The new quality control methods for detecting PEI amount on PEI-cloth and monitoring the quality of PEI cloth are described.

Kouchi Matsumoto, Masayo Sakata, Naoko Iwahashi, Masashi Kunitake,

Masao Miyazaki, Chuichi Hirayama
"Poly(ethylenimine)-immobilized-cloth enzyme immunoassay for the detection of *Salmonella* lipopolysaccharide", *Immunological Invesgations*, **32** (1&2), 3-14 (2003)

Kouchi Matsumoto, Masayo Sakata, Naoko Iwahashi, Masashi Kunitake, Masao Miyazaki, Chuichi Hirayama
"Detection of *Salmonella* LPS by poly(ethyleneimine)-immobilized-cloth enzyme immunoassay", Proceedings of '01 Kyushu-Seibu/Pusan-Kyeongnam Joint Symposium on High Polymers (10th) and Fibers (8th), Nagasaki, Japan. pp. 161-162. November 1-3, 2001

Chapter Four describes about the development of CEIA searching for the suitable conditions of the assay using p-cloth and PEI-cloth with TMB reagents and blocker-stabilizer-diluent reagent. For successful CEIA, the amount of PEI on the cloth was found to be very crucial and thus the relationship between PEI amount and the resulting CEIA was studied. Then p-cloth and PEI-cloth for CEIA use were compared. Using PEI-cloth, a method to detect minute amount of LPS in large volume sample through concentration is described.

Kouchi Matsumoto, Masayo Sakata, Naoko Iwahashi, Masashi Kunitake, Masao Miyazaki, Chuichi Hirayama
"Poly(ethylenimine)-immobilized-cloth enzyme immunoassay for the detection of *Salmonella* lipopolysaccharide", *Immunological Invesgations*, **32** (1&2), 3-14 (2003)

Kouchi Matsumoto, Masayo Sakata, Masashi Kunitake, Masao Miyazaki, Chuichi Hirayama
"Rapid detection of a minute amount of lipopolysaccharide from *Salmonella* in large volumes by poly(ethyleneimine)-cloth enzyme immunoassay"
Biotechnology Letters, **24**, 1791-1795 (2002)

Kouchi Matsumoto, Masayo Sakata, Masashi Kunitake, Masao Miyazaki, Tetsuhide Inoue, Chuichi Hirayama
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Japanese Patent, Applied No. 2001-332136, applied on Oct. 30 (2002)

Kouchi Matsumoto, Masayo Sakata, Naoko Iwahashi, Masashi Kunitake, Masao Miyazaki, Chuichi Hirayama
"Preferential adsorption of Salmonella lipopolysaccharides on poly(ethyleneimine)-cloth applied to enzyme immunoassay" 22nd International Symposium on the Separation of Proteins, Peptides and Polynucleotides, Heidelberg, Germany. Book of Abstracts, p. 44, Nov. 10-13, (2002)

Chapter Five describes the successful preparation of new adsorbent PEI spherical beads and as possible new LPS and nucleic acid removing adsorbent. The preferential adsorption of LPS and DNA was found to be a useful method to remove minute amount of contaminated LPS and recombinant DNA from medical use fluids of biological origin without inactivating and removing the ingredients.

Masayo Sakata, Kouchi Matsumoto, Nagi Obaru, Masashi Kunitake, Hiroshi Mizokami, Chuichi Hirayama
"Removal of DNA from a protein solution with cross-linked poly(ethyleneimine) spherical particles", *Journal of Liquid Chromatography & Related Technologies*, **26** (2), 227-242 (2003)

Kouchi Matsumoto, Masayo Sakata, Nagi Obaru, Masashi Kunitake, Masao Miyazaki, Chuichi Hirayama
"Effect of amino group content of cross-linked poly(ethyleneimine) spherical adsorbents on removal of DNA from protein solutions", 22nd International

Symposium on the Separation of Proteins, Peptides and Polynucleotides (ISPPP 2002), Book of Abstracts, p. 43, Heidelberg, Nov. 10-13, 2002.

Masayo Sakata, Kouchi Matsumoto, Takashi Kamada, Masashi Kunitake, Chuichi Hirayama
"Cross-linked N,N-dimethylaminopropylacrylamide spherical particles for removal of nucleic acid from protein solution"
Polymer and Organic Chemistry, University of California San Diego, La Jolla, CA, USA, Book of Abstracts, Abstract Number 37 (2 pages), July 14-18 (2002)

In **Chapter Six**, the results of all previous chapters were summarized and concluded.

In above studies, using PEI in the forms of cloth and spherical beads, PEI showed very unique and useful capabilities as specific adsorbing material for LPS and DNA. PEI-cloth together with new formulation of substrate TMB reagent, enzyme-antibody stabilizer and blocker provided a very sensitive, economical, safe and easy to use enzyme immunoassay. This method may greatly contribute to monitor and prevent epidemics and hygienic in food manufacturing and so on. PEI spherical bead is found to be a very efficient remover of LPS and DNA, which will contribute to produce safe vaccines and blood products with minimum of such contaminants.

Chapter 1. **Introduction**

1.1. **Endotoxin**

Endotoxin was first described in 1892 by Pfeiffer (1) about a heat stable toxin released only from a disrupted or disintegrating cell of *Vibrio cholerae*, while exotoxin is proteineous toxin produced and released by the cell. Since then such endotoxin was widely found amongst Gram-negative bacteria cell wall such as *Echerichia, Salmonella, Shigella, Klebsiella, Pseudomonas* etc (Fig. 1-1). When our body is exposed to endotoxin excessively or systemically, a systemic inflammatory is caused and leading to multiple organ failure, shock and potentially death even at nano gram level. Weastphal et al (3) successfully separated endotoxin as lipopolysaccharide (LPS) free of protein from intestine bacteria by hot phenol-water extraction method. Further they hydrolyzed LPS to find that the obtained hydrophobic material consisted of D-glucosamine, phosphate and lipids connected by ester and amide linkages, and named as lipid A showing that the lipid A is the highly hydrophobic and endotoxic active part of the molecule (4) as shown in Fig. 1-2. & 1-3. (5) (6).

Presence of hazardous food pathogens such as *Salmonella, Escherichia coli* 0157:H7, *Campylobacter jejuni* and their endotoxins greatly influence on our health and life. A new food safety measure known as "HACCP" (Hazard Analysis Critical Control Point) system is widely implemented among food manufacturing sectors in industrial countries. This system is to monitor the hazard levels at critical control points of food processing to take measures microbial pathogenic contamination to reduce or eliminate hazards. As the implementation of HACCP increases, microbial tests may increase, which costs will add to significantly on the cost of foods and effect to the competitiveness of its trade. Thus the test should be simple, no biohazards generated especially performed in food processing plants, cost-effective, rapid, accurate and safe. For such purpose and demands, the enzyme immunoassay for the endotoxin detection method is a quite reasonable choice. This applies to biomedical research as well.

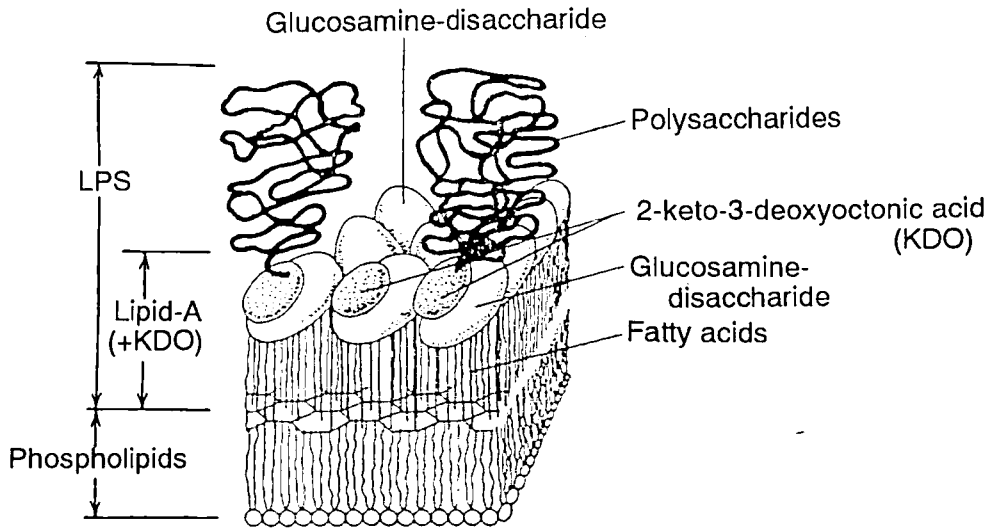


Fig.1-1. Schematic structure of Gram-negative bacteria outer membrane (2)

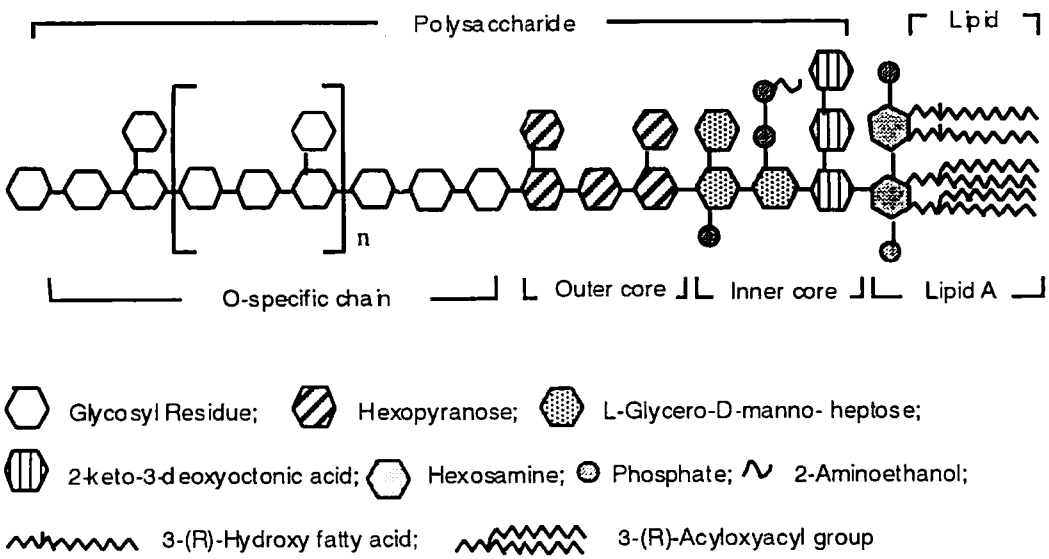
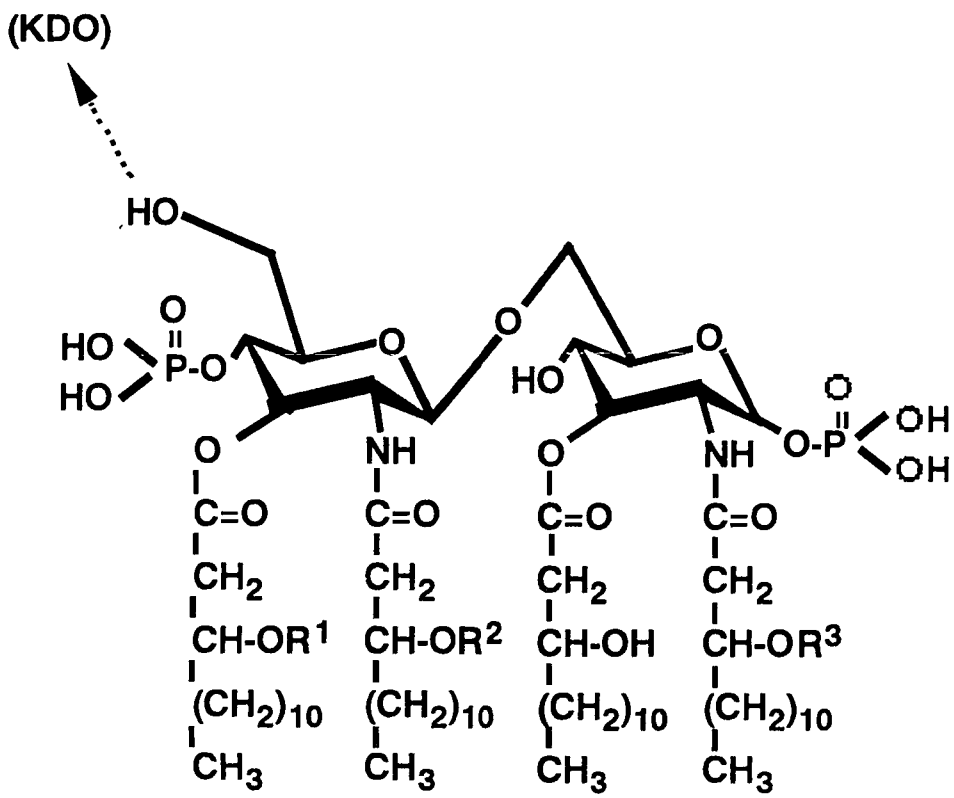


Fig. 1-2. Architecture and schematic structure of an endotoxin from enterobacteriaceae (4)



Salmonera minnesota : $R^1 = C_{16}, R^2 = C_{12}, R^3 = C_{16}$

Escherichia coli : $R^1 = C_{16}, R^2 = R^3 = C_{16}$

Pseudomonas mirabilis : $R^1 = H, R^2 = C_{12}, R^3 = H$

$C_n = CH_3(CH_2)_{n-2}C(O)$ -

Fig. 1-3. Chemical structure of various Gram-negative bacterial lipid A. Modified after Seydel et al. (6). KDO: 2-keto-3-deoxyoctonic acid.

1.2. **Endotoxin detection method**

1.2.1. **Enzyme Immunoassay (EIA)**

Developing methods to detect pathogens of infectious diseases in clinical specimens, food, and food processing tools or as environmental pollutants is an important area of biotechnological and biomedical research. The enzyme-linked immunosorbent assay (ELISA) or capture enzyme immunoassay (EIA) is a well utilized practical method for this purpose (Fig.1-4). In animal blood, antibody (protein) is formed by certain white blood cells in response to the entered foreign substances as antigenic substances (such as pathogens, toxins, pollen, pesticides and herbicides as organic pollutants, foreign blood and tissues etc.) to render harmless. The reaction of antibody to antigen is quite specific and called immune response. EIA utilizes the antibody specificity to particular antigen which is to be detected. The capture EIA is carried out by adsorbing the antibody onto a microwell, a solid flat support made of polystyrene, and is commonly known as the microwell-based capture EIA (well-EIA) method (Fig.1-4 b). The EIA can also be performed on a microporous membrane usually made of nitrocellulose or poly(vinylidene fluoride) (PVDF), and then the assay is known as the dot blot method (Fig.1-4 a). This way, antibody adsorb onto a solid matrix and it captures the specific antigen that is to be detected in the sample. The captured antigen is revealed by subsequent addition of second antibody specific to the second site of the test antigen, which is conjugated with an enzyme such as horseradish peroxidase (HRP). When the substrate of the enzyme is added, a color signal is produced and its intensity corresponds to the concentration of antigen captured. This color signal is measured spectrophotometrically in microwell method and as color intensity spot in dot blot method.

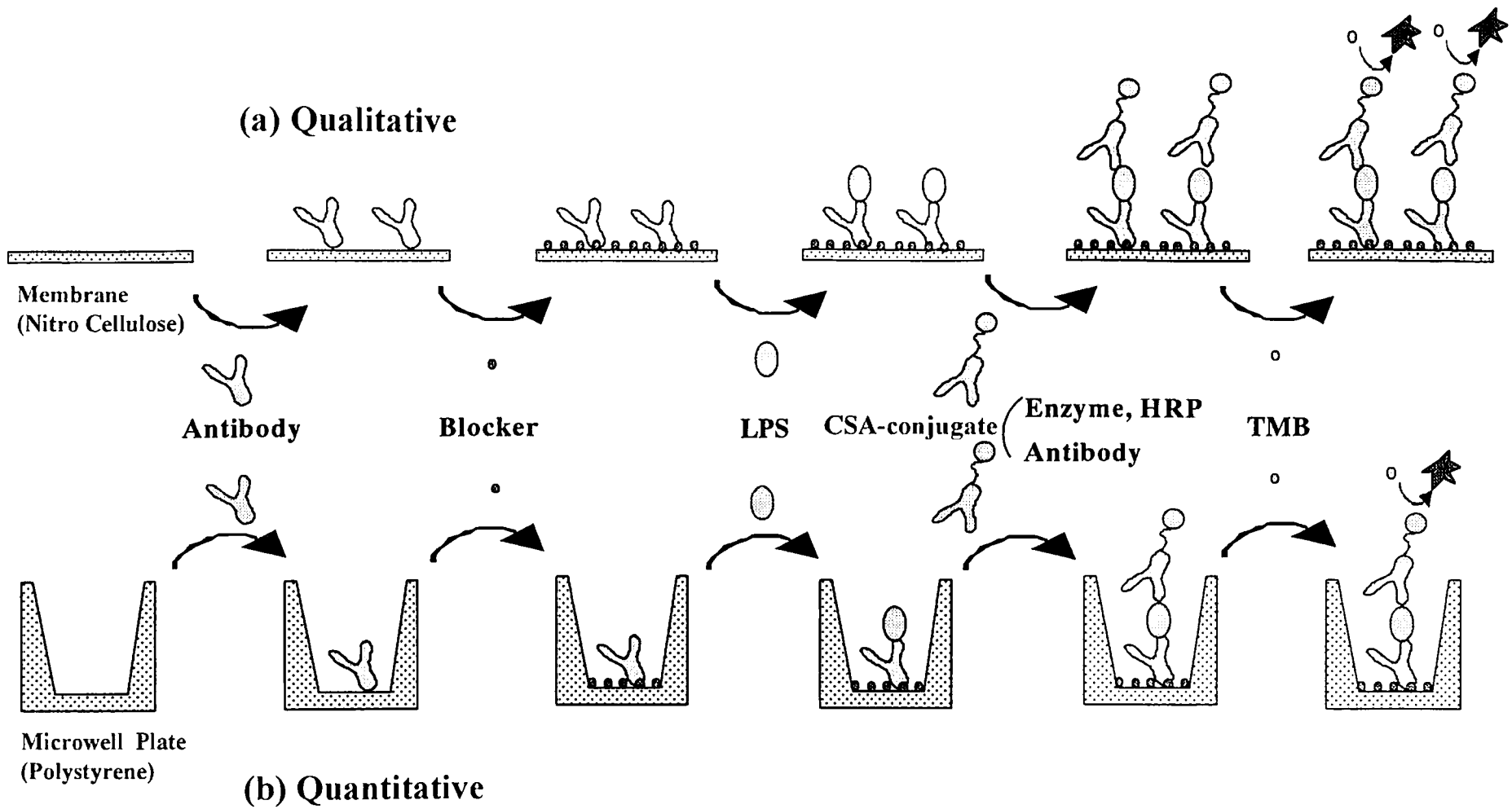


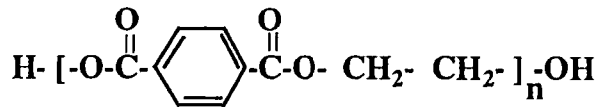
Fig.1-4. Enzyme-Linked ImmunoSorbent Assay (ELISA)

1.2.2. **Development of cloth enzyme immunoassay (CEIA)**

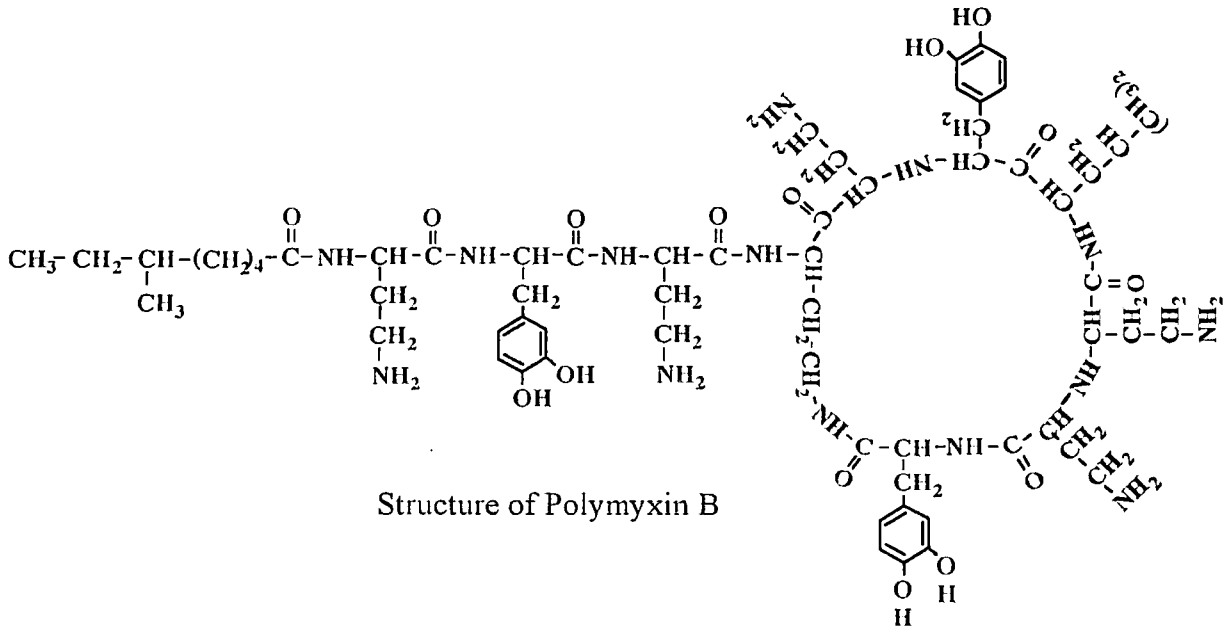
Modifications to develop EIA that are more rapid, reliable, safe and economical to detect various pathogens, pollutants, toxins are constantly being developed. Blais and Yamazaki (7) (8) (9) developed a cloth enzyme immunoassay (CEIA) to detect pathogenic bacteria. The method uses polymyxin B coated non-woven polyester cloth as the macroporous solid support, on which the Gram-negative bacteria's lipopolysaccharide (LPS) is selectively captured. Polymyxin B was utilized in this EIA based on its affinity for LPS. Polymyxin B's affinity for LPS had previously been utilized in a commercially prepared polymyxin bound cross-linked agarose gel marketed to remove LPS from solutions (10).

The selective binding for LPS can be explained from the fact that polymyxin B is a surface active cyclic peptide antibiotic (Fig. 1-5) that has bactericidal activity against Gram-negative bacteria by insertion of hydrophobic tripeptide, fatty acyl peptide, into the cell wall to break down through interacting with lipid A portion of LPS (11).

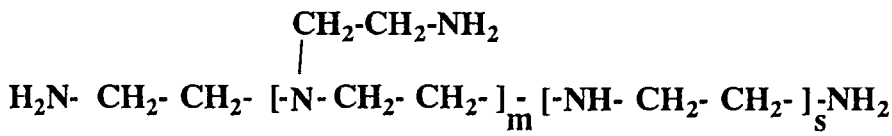
Polymyxin B is adsorbed through hydrophobic and hydrogen bonding on non-woven polyester cloth which contains terephthalate groups in the structure. Polymyxin on the cloth captures the lipid A region of LPS. For the specificity, controls were performed utilizing two model hydrophobic compounds, deoxycholate and caprylic acids, and these compounds caused no change in the CEIA signal, indicating that the reaction seen with LPS was not due to a non-specific hydrophobic interaction (7).



Structure of Polyester



Structure of Polymyxin B



Structure of PEI

Fig. 1-5. Structures of polyester, polymyxin B and PEI

1.2.3. **Poly(ethyleneimine) (PEI) as a specific LPS adsorber**

In studies of LPS capturing materials, polymers with amine groups utilized for removal of LPS in medicine and biological-origin fluids are often prepared in bead form by controlling the pH, ionic strength and pore size (12) (13). Among those amine-contained polymers, poly(ethyleneimine) (PEI) has been reported to react preferentially with LPS (14) (15). This may be due to the similar functional groups between PEI and polymyxin possessed in the structures, eg. primary, secondary, tertiary amine groups and short aliphatic groups, and pKa 8.7 while 9.2 for polymyxin B. These studies indicates that ionic bonding between the amino groups of polymyxin or PEI and the phosphate groups of LPS, could be involved besides the hydrophobic interactions.

This specific binding is biologically confirmed as well that polycationic molecules such as PEI disrupt the integrity of outer membrane (OM) in loss of the barrier function but lacking direct bactericidal activity. The aliphatic and cationic polymer substance PEI binds to OM of Gram-negative bacteria and sensitize to detergent-induced lysis leading to death of the cell(16) and hydrophobic antibiotics Polymyxin B nanopeptide is found to act similarly (17). These agents bindings to OM caused considerable alteration which were visualized by electronmicroscopy (18) (19). Other polycationic substances such as protamine and certain polylysines disrupt the OM with concomitant release of major portion of LPS from the cells (17). Now PEI is available commercially with variety of molecular weights and can be chemically bonded or adsorbed onto polyester cloth in a manner similar to polymyxin B through hydrophobic and hydrogen bonding. We expected that replacing polymyxin B with PEI and develop PEI-CEIA retaining all the advantages of polymyxin-CEIA (20).

1.2.4. **EIA substrateTMB**

In enzyme immunoassay, enzyme itself or enzyme-antibody conjugate is used to have enzymatic reaction with chromophore substrates to quantify

and identify the presence of target antigen. For this purpose, stable marker enzymes such as horseradish peroxidase (HRP), alkaline phosphatase and β-galactosidase are commonly used, but HRP is the most popular one because of the best sensitivity in colorimetric assays among them and a safer substrate is available (21). Among traditional substrates of HRP are o-phenylenediamine (OPD), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS), benzidine and 4-chloro-1-naphthol (CN) but they are all mutagenic or carcinogenic as the serious drawback (22). However the derivative of benzidine, 3,3',5,5' tetramethyl benzidine, (TMB) (Fig. 1-6) turned out to be an excellent substrate of four fold as sensitive as benzidine and others but poorly soluble and decomposes in water (23) yet neither carcinogenic nor mutagenic (24). TMB stock solutions prepared in dimethylsulfoxide or methanol/acetone or ethanol can last only for a few weeks (25) and rapidly form dark brownish color and lose up to 20% TMB content (26). Besides the low stability, the poor solubility causes a problem of the rate limmiting factor for EIA. Thus we focused on this substrate to develop to be a water soluble reagent in a form of powder because of the non-carcinogenicity, non-mutagenicity and high sensitivity.

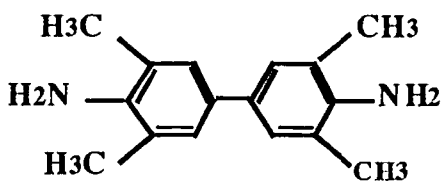


Fig. 1-6. TMB structure

All commercially available TMB reagents are in liquid form to be kept in refrigerator and the solvents are often DMSO or DMF which are not safe and environmentally sound. For commercial dealer, they have to ship in a bulky box with cooling aid which is not economically and environmentally favorable either. To overcome these problems, a powder form of TMB

reagents was prepared that is water-soluble, highly sensitive, environmentally safe and stored taking only small space being stable for long period of time at room temperature (27).

1.2.5. **Blocker and stabilizer in EIA**

In EIA, it is important and one of the most crucial steps to optimize assay but quite often overlooked field is blocking. High background in immunoassay is caused by nonspecific interactions between assay components and the solid surface (Fig. 1-7a). More specifically, a blocker functions to block of antibody or antibody-enzyme conjugate from adsorbing on a solid surface and helps to retain only specific bindings so that it reduce or eliminate the amount of background caused by the non-specific bindings, but has no active part in immunoreactions. As a result, it improves assay sensitivity. It is usual practice to pre-coat the solid surface (Fig. 1-7b). Among commercially available blockers are BSA, powder skim milk, Casein. These protein blockers and ethylene glycol work to stabilize enzyme, antibody and enzyme-antibody conjugate as well when stored at low temperature. During assay procedure, they have to be diluted and incubated at higher temperature such as 20-37℃, which causes losing activity over the time. Thus it is important to preserve the conjugate activity for long period of time even at ambient temperature. It is not exaggeration to say that the success of an enzyme immunoassay system is quite depended on choosing proper blocker and stabilizer. Thus a blocker and stabilizer can be contained in enzyme-antibody conjugate solution as a diluent instead of pre-coat the solid surface in recent EIA practice (Fig. 1-7c).

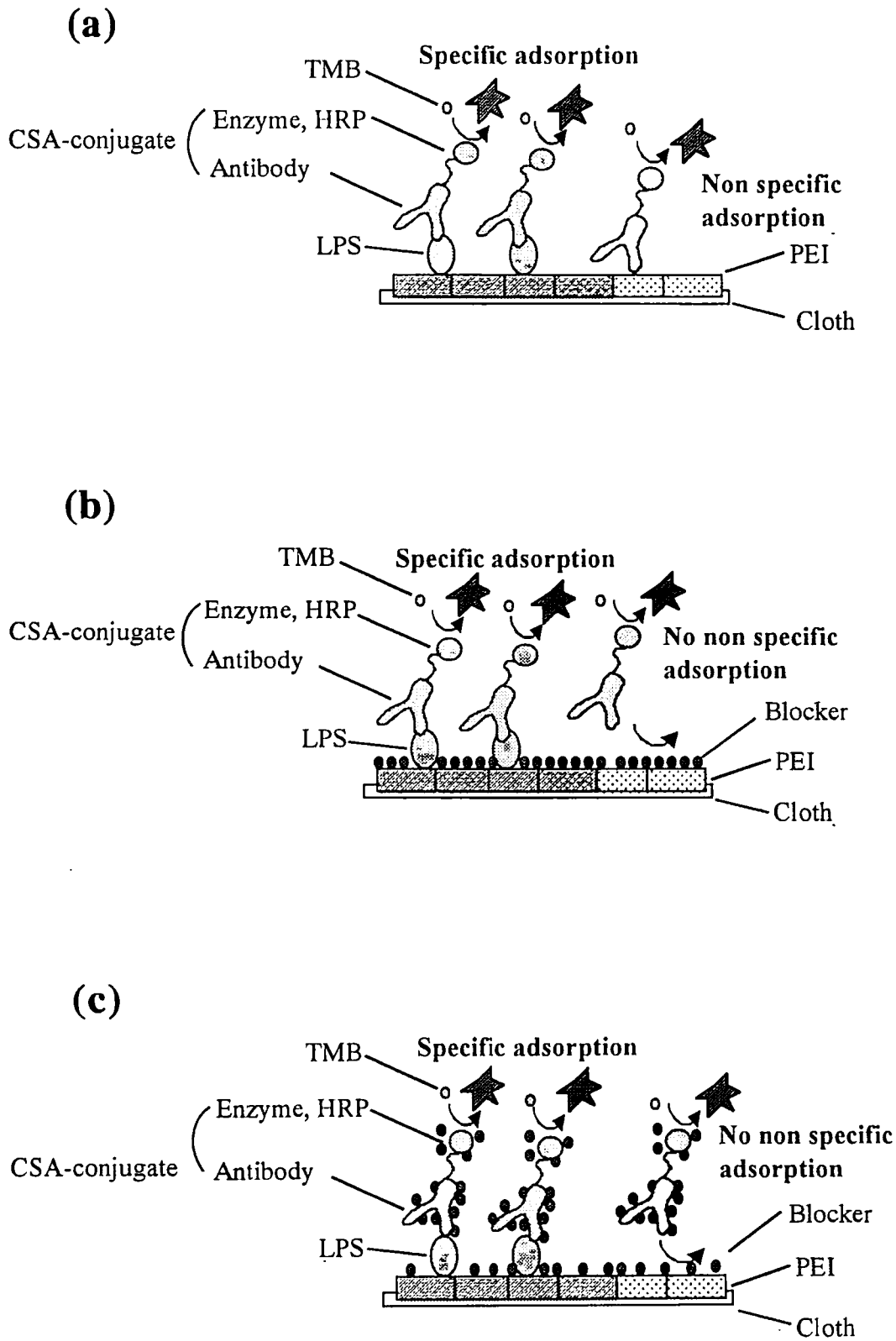


Fig. 1-7. Function of Blocker

1.3. **Selective removal of endotoxin by adsorption methods**

Removal of endotoxin from medical fluids for injections including vaccines must fulfill many required conditions. Such medical agents contains rather unstable high molecular weight proteins and organic compounds as active ingredients which should not be removed nor chemically modified nor destroyed by the method. Also the removal method should not shed biohazard compounds behind but must effectively remove LPS to below 5 endotoxin units (EU) per kg body weight an hour set by all pharmacopoeias for intravenous applications as specified (28). Here, 1 EU corresponds to 100pg of the standard endotoxin EC-5 or 120 pg of endotoxin from *Escherichia coli* O111:B4 (29). For this purpose, various removal methods such as ion-exchanger on membrane (30) or filter (31), ultrafitration (32) (33), extraction (34) (35) and sucrose gradient ultra-centrifugation (36). However these methods are unsatisfactory with respect to LPS selectivity, adsorption capacity and recovery of the protein products. Such specific and gentle way to remove LPS from unstable medical fluid is suited with specific LPS adsorption method. Several spherical beads such as aminated poly (γ-methyl L-glutamate) (PMLG-NH_2) spheres (37), cross-linked N,N-dimethylaminopropylacrylamide (DMP) spherical particles (38) and poly (ε-lysine) immobilized cellulose particles (PL-cellulose) (39) were prepared and gave very promising LPS selectivity and capacity. With similar idea, cross-linked PEI was prepared and studied for the LPS removal because it has low toxicity, easily available in low cost and known for the selectivity on LPS (40).

1.4. **Selective removal of DNA by adsorption methods**

In the biotechnology industry, various continuous cell lines, such as the Chinese Hamster Ovary cell line, are widely used for producing recombinant DNA products such as protein and protective antigen. These cellular products are always contaminated with their oncogenes in the residual host cell DNA. Therefore, such contaminants have to be removed

from biopharmaceutical product solutions used for intravenous administration, because of concerns about the possibility of cellular transformation by this potentially oncogenic DNA (41). In 1997, a World Health Organization consultative group recommended that the safety risk was negligible or non-existent in products that contained less than 10 ng per dose of cellular DNA (42).

To remove DNA from protein solutions, the selective adsorption method has proven to be most effective. In order to achieve a selective removal of DNA from solutions of high-molecular-weight compounds, such as proteins, by using adsorbents, it is necessary to consider not only the chemical and physical structures of DNA but also those of adsorbents and proteins, and the solution conditions. In physiological solutions, DNA molecules exist in a wide range of sizes, from M_w $1x10^4$ to $1x10^6$, or larger. On the other hand, the molecular weights of proteins are generally about $1x10^4$ to $5x10^5$. Therefore, it is extremely difficult to separate DNA from protein only by size-separation methods such as size-exclusion chromatography or ultrafiltration.

Already it has been reported that various cationic polymer adsorbents, such as histidine-immobilized Sepharose (43) and Chitosan particles (44.45), were very useful as adsorbents for anionic bio-related polymers, such as LPS and nucleic acids (DNA and RNA). However, it has been reported (43) that these adsorbents cannot selectively removed DNA from acidic protein solutions, such as BSA, because of their high adsorption for both DNA and BSA. Therefore it was attempted to develop DNA adsorbent capable of retaining high selectivity for DNA under physiological conditions. It has been found that aminated poly(γ-methyl L-glutamate) (PMLG) spherical porous particles showed superior DNA selectivity than commercial DNA adsorbents based on chitosan (46). However, a great disadvantage of this type of adsorbent is the low chemical stability of an ester bond originating from the side chain of PMLG (47). Thus, their regeneration at high and low pH is ruled out.

In this part of work, cross-linked poly(ethyleneimine) (PEI) spherical particles as a novel adsorbent were developed, from PEI and chloromethyloxirane (CMO). The PEI has the characteristics of a polycation with long and flexible chains of amino-groups, and has higher pK_a (8.7) than chitosan (pK_a: 6.2). E. Unsal et al. reported (48) that PEI-attached poly(*p*-chloromethylstyrene) beads showed high DNA adsorption, and that they were suitable materials for the immobilization of DNA. Thus, it is expected that the cross-linked PEI particles also show high DNA-binding activity. The particles, being composed of –CHNH- bonds by the cross-linking of PEI and CMO, are also expected to keep their entire structure in solutions of high and low pH.

1.5. **Aim of the thesis**

Gram-negative bacteria such as *Salmonella, E. coli, Campylobacter, Vibrio vulnificus, Staphylococcus, Bacillus cereus, Listeria monocytogenes, Yersinia enterccolitica* etc are serious foodborne pathogens. It is crucial to detect these pathogens to prevent outbreaks. Thus it is desired to develop such an enzyme immunoassay method that is sensitive, economical and easy to use. New type of PEI-CEIA and accompanying reagents were developed to fulfill this goal. Also a very minute amount of LPS contaminated in a sample can be detected by concentration utilizing the macroporous nature of cloth. On the other hand, LPS is pyrogenic and may cause endotoxin shock to death if it is introduced in blood. Thus, its removal from medicine, injection solution, blood products and vaccination products is required. Removal of recombinant DNA from vaccine prepared in bacteria is desired even though the true consequences for human are not known yet. New adsorbent PEI spherical beads were designed and prepared. The preferential adsorptions of LPS and DNA were found to be useful to remove minute

amount contaminated LPS and recombinant DNA from medical use fluids of biological origin.

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Chapter 2. **Development of new formulation of enzyme-antibody conjugate substrate and preparation of a blocker-stabilizer-diluent agent for the enzyme-antibody conjugate in EIA**

2.1. **New formulation of enzyme-antibody conjugate substrate**

In enzyme immunoassay, enzyme-antibody conjugate is used to have enzymatic reaction with color forming chromatic substrates to quantify or qualify to identify the presence of target antigen. For this purpose, stable enzymes such as horseradish peroxidase (HRP) and alkaline phosphatase are commonly used, but the former is more popular because of better sensitivity, less costly and safer substrate available. A substrate of horseradish peroxidase is TMB which is highly sensitive and neither mutagenic nor carcinogenic but insoluble in water (1) (2). We focused on this substrate to develop a reagent formula that is in powder form because it does not occupy storage space and stable at room temperature for long period of time and yet soluble in water.

2.2. **Development of powdered formulation of soluble and insoluble TMB reagents as the substrates for HRP**

The powder form of reagent will occupy less space in the laboratory and stored at room temperature, and for shipping, takes less volume, weight and cost than the liquid form. There are two forms of TMB reagent which are called as soluble and insoluble TMB reagent. The captured antigen is revealed by subsequent addition of antibody-enzyme conjugate and the substrate of the enzyme, then a color signal is produced and its intensity corresponds to the concentration of antigen captured. When performing the well-capture EIA, a soluble substrate is used and the soluble end product is determined by a spectrophotometer. The dot blot method utilizes an insoluble substrate in which the end product precipitates making stains on the membrane or cloth as a spot. Based on this, the two differently formulated substrate reagents are commonly named "soluble" or "insoluble" TMB reagent.

In HRP conjugate assay system, because TMB itself does not dissolve in

water, it is common practice to dissolve it in organic solvent (e.g., ethanol) before it becomes miscible in an aqueous solution to prepare as the substrate. The water soluble Hcl salt is available but may effect on pH. To keep the reagents stable for a long time, commercial reagents usually have two separate solutions, TMB solution and other ingredients including peroxide solution, and mix them before use, but some are one reagent system with some stabilizers. and they are stable for about 1 year in refrigerator. Our aim is to prepare a water soluble, powdered form of stable TMB reagents consisting of water dissolvable water-insoluble chromogenic agent TMB mixed with other powder ingredients. To prepare water-soluble TMB in powder form, following characteristics of a suitable polymer to mix were considered,

1) dissolve in water quickly
2) the hardness is reasonable for easy powdering and not sticky
3) its molten form can dissolve TMB and does not decompose itself and TMB at high temperature heated around 110~130 ℃.
4) no negative effect on the activities and stabilities of the enzyme and antibody during assay
5) no chemical reactions with other mixing ingredients in TMB reagents
6) during assay, neither forming precipitation nor influence on spectrophotometric absorbency
7) economical and environmentally safe
8) as part of TMB reagent, it stabilize for long shelving at room temperature

Among water soluble and heat stable polymers to fulfil above requirements, polyethylene glycol (PEG) and polyvinyl alcohol (PVA) could be suitable but PVA works as a blocker in assay, which in this case was not suitable. PEG is well used in enzyme field as a stabilizer and has amphipathic nature. Thus PEG and TMB could form complex through ethylene residue with aromatic hydrocarbon hydrophobically and ether oxygen and end OH group forming hydrogen bonding. Thus TMB dissolved in molten PEG and the solidified complex of TMB with PEG dissolved in water (Fig. 2-1). Among commercially available PEG, below molecular weight of 600 is liquid or paste. PEG between

1000 to 10000 is found to be solid enough and easy to melt and powder. Especially MW 3350 was found easy to handle in powdering with right hardness for this purpose.

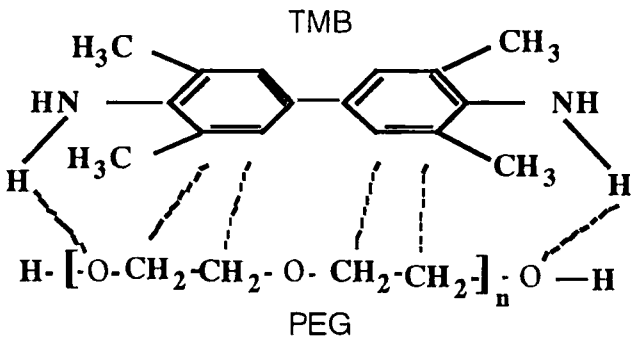


Fig. 2-1. Complex forming between TMB and PEG

2. 3. **Preparation of water soluble TMB**

2.3.1. **Materials**

Following products were obtained from Aldrich Fine Chemicals. 3,3',5,5' tetramethyl benzidine, (TMB); 86,033-6. Poly(ethylene glycol) MW 3400 (PEG); 20,244-4. Citric acid monohydrate; 45,405-2. Ethylenediaminetetraacetic acid, tetrasodium salt dihydrate (EDTA); Sigma ED455. Dextran sulfate sodium salt; Sigma D 7037. Sodium perborate monohydrate; 37.286-2.

2.3.2. **Small scale preparation**

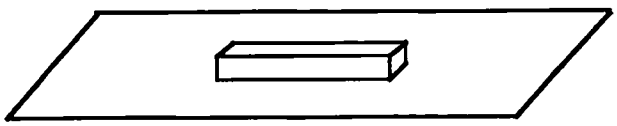
PEG 3400 powder 3g was placed in a 20 ml vial without cap and heated in a block heater at 110-120°C to release any volatile materials (eg, water) for 2 hr with occasional swirling then lowered temperature to 80 degree and added TMB powder 12 mg in portions. Added TMB and PEG were in the weight ratio of 1 to 250. It was mixed well by swirling and TMB was completely dissolved. The mixture was continued heating at this temperature for 2 hr. Then it was cooled

down to room temperature and powdered. The solubility in water was tested. Among different TMB/PEG ratios tested for the complete solubility, 1 to 200 ratio product was soluble in water but occasionally formed precipitation after standing. On the other hand 1 to 250 ratio had always a clear solution. It was found that water insoluble CN become soluble in water by this method (2).

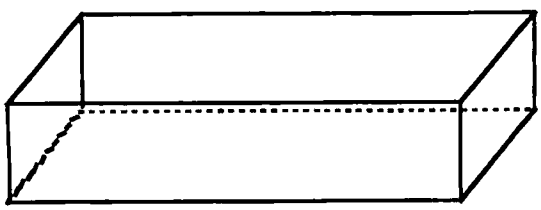
2.3.3. **Pilot plant scale preparation**

For larger scale preparation, a stainless steel box (10.3 x 23 x 6 cm) with a flat stainless cover (11.3 x 24cm) was fit into the well of a block heater (3 block size. Thermolyne Dry-bath by Sybron/Thermolyne) without blocks as seen in Fig. 2-2.

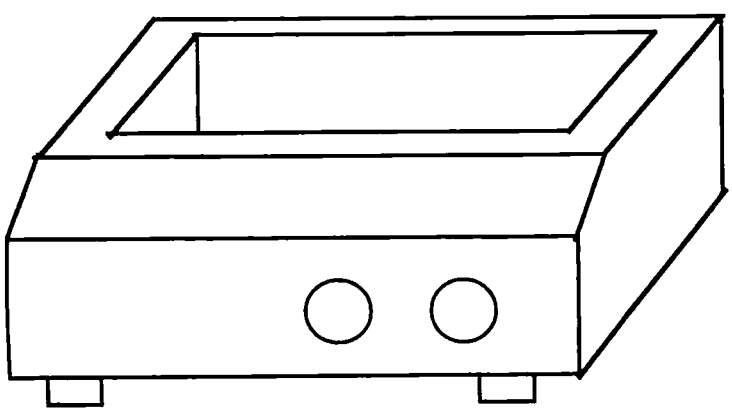
In the vessel 500g of PEG 3400 powder was placed evenly, then heated to 110-120°C with occasional mixing with spatula and monitoring the temperature with a thermometer for 2 hr without cap. Then the temperature was lowered to 80°C and TMB powder 2 g was added portion wise by spreading around over the surface and mixed with spatula to dissolve completely and evenly. After complete dissolve, heating was continued at 80°C for another 2 hr with cap on. The vessel was carefully placed in a cold water bath and let it solidify. The cake was cut to about 0.5 cm small cubes with chisel. In fume-hood of low moisture environment, it was then powdered in a well sealed blender (eg., Osterizer 14 speed Heavy duty Blender). The powder was sieved with Tyler 32 mesh (500μm) and repeatedly blended till all powder passed through. As TMB is sensitive to moisture, the powder was stored tightly sealed from moisture and UV light in a brown bottle (It is advisable to keep with a package of silica gel drying agent) for long storage at room temperature.



Stainless cover



Stainless vessel



Block heater

Fig. 2-2. Block heater and the stainless steel vessel to disolve TMB in molten PEG.
The vessel was used for solidify the mixture and the TMB/PEG cake was powdered by a blender.

2.3.4. **Other components of TMB reagent**

It is important that TMB/PEG mixed with other reagent components does not form precipitation nor color and effect to neither enzyme nor antibody activity.

2.3.4.1. **Preparation of buffer powder**

The buffer powder should not easily absorb moisture and keep pH 7.2 for HRP. Among buffers tried, phosphate buffer easily absorbed moisture and made powder TMB reagent to form lumps and blue color. Citrate buffer worked well as the buffer but occasionally made TMB reagent form light yellow color during storage. Citrate-EDTA buffer gave good storage as far as it was kept dry. Both reagents citrate and EDTA tetra sodium were powdered by motor and pestle and well mixed. The final buffer recipe was citrate: EDTA=1:2 mixture by weight.

2.3.4.2. **Preparation of dextran sulfate.**

Dextran sulfate prepared from dextran with MW of 5000 was suitable. It captured color products of TMB and stuck to matrix forming a blue color spot in dot blot (insoluble TMB). Some batch of commercial dextran sulfate become brown color after opened due to decomposition and forming sulfuring acid. It was recrystallized from alcohol. The dried and powdered dextran sulfate was used for the preparation of insoluble TMB reagent. Cellulose sulfate was usable for this purpose but available products were not reliable by lot because they were not sieved by molecular weights and not sulfonated enough to be soluble in water, which consequently formed precipitation.

2.3.4.3. **Preparation of sodium perborate**

In the HRP system, electron accepter H_2O_2 must be present but has to be in solid form. Sodium perborate ($NaBO_3$) was found to be suitable. Usually available in fine beads form and was mixed as it was.

2.4. **Formulation and usage of powder TMB reagents**

2.4.1. **Preparation of soluble TMB reagent**

Prepared powder agents as above were mixed as follows;

TMB/PEG powder	5 g
Citrate-EDTA buffer	0.76 g
Na BO_3	0.04 g

2.4.2. **Preparation of insoluble TMB reagent**

Prepared powder agents as above were mixed as follows;

TMB/PEG powder	5 g
Citrate-EDTA buffer	0.76 g
Dextran sulfate	0.20 g
Na BO_3	0.04 g

Those mixtures should be homogeneously well mixed and kept at room temperature in a brown bottle and away from moisture (keep with a package of drying agent). For usage, 35 mg of above powder was dissolved in 1 ml double distilled water (3). The powder reagents were stable at least for 7 years stored at room temperature in dry atmosphere.

2.4.3. **HRP catalyzed oxidation of TMB color forming reaction by TMB reagent**

In the above powder TMB reagents, the chromogen TMB works as the electron donor and hydrogen peroxide from sodium perborate as the electron acceptor. It is said that ~95% of the perborate correspond to 9.9% available oxygen. In case of soluble TMB reagent, the enzymatic reaction is terminated by addition of 2N sulfuric acid which results in shift of pH to below unity and complete conversion of the blue oxidation product to yellow diamine form which can be quantified by measuring absorbency at 450 nm as presented in Fig. 2-3. In case of insoluble TMB reagent, the blue color product is caught by dexstran sulfate on the matrix (cloth) and excess of TMB is washed away instead of addition of sulfuric acid to terminate the reaction (4).

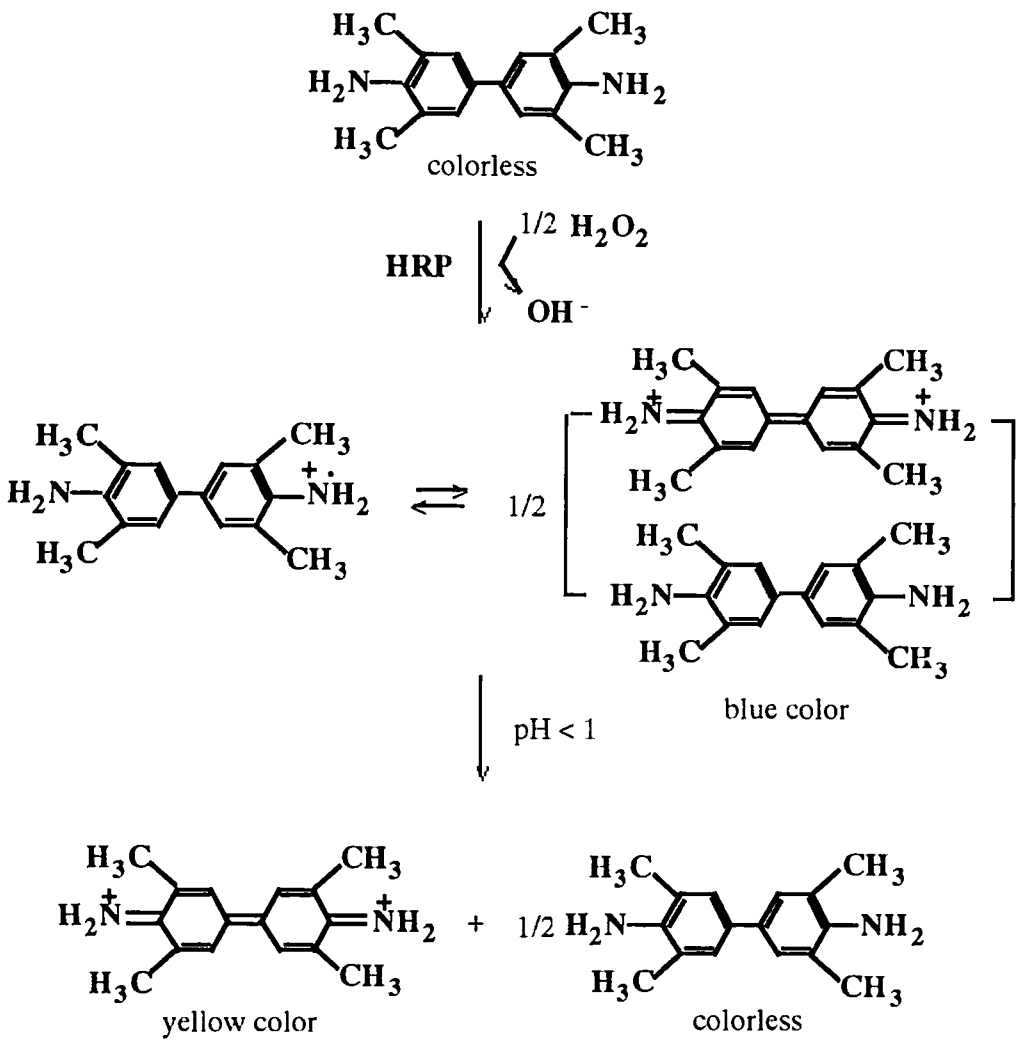


Fig. 2-3. HRP catalized oxidation of chromophore TMB to give color

2.5. Development of a unique blocking-stabilizing-diluent reagent for CEIA

2.5.1. Blocker and stabilizer in EIA

Blockers works by blocking the nonspecific bindings of conjugate to matrix to reduce back ground. Conventional blockers are bovine serum albumin (BSA) (5), casein, normal goat serum (6), skim milk and sheep IgG (7) for example. As a synthesized blocker, polyvinyl alcohol works as a blocker for horseradish peroxidase (8). Each assay system has to establish which blocker to use and the concentration etc for the best assay conditions to obtain the largest peak to noise ratio which means to attain best sensitivity. It is a usual practice to have a step to pre-coat a blocker on the matrix before applying samples but it was found to work same by contained as a diluent of the conjugate. It is important to stabilize the enzyme-antibody conjugate because it is not stable enough but expensive, otherwisethe reproducibility and reliability of EIA decline. Conventional stabilizers are BSA, casein, serum and ovalbumin. There are some synthesized stabilizers. It was found that polyvinyl alcohol stabilize horseradish peroxidase and glucose oxidase (9) (10). The water-soluble amphiphilic phospholipid polymer, poly[2-methacryloyloxyyethyl phosphorylcholine (MPC)-co-stylrene(St)] stabilized horseradish peroxidase conjugate (11). These synthesized stabilizers were prepared to solve the problem of losing activity by repeated freeze and melt.

2.5.2. Blocker which stabilize HRP conjugate and use as diluent as well

Blocker was used by pre-coating the matrix, but now contained as diluent in the solution of antibody or antibody-enzyme conjugate rather than pre-coating (12). A fraction extracted from powder skim milk found to have good blocking and stabilizing effect. This fraction can be included in the solution of antibody or antibody-enzyme conjugate, thus it is working as a diluent. The blocker-stabilizer-diluent was developed to preserve the activity of enzyme-antibody conjugate even stored at room temperature or above for long period of time as far as presence of some preservative such as 0.1% Thimerosal so that the

conjugate can be safely shipped at ambient temperature or do field work without using ice or freeze as used to be. Such fraction separated from skim milk had especially good stabilizing effect on HRP-conjugate and the extraction method is presented as follows.

2.5.3. **Preparation of Blocker-Stabilizer-Diluent from skim milk powder**

2.5.3.1. **Materials and preparation of buffers**

Powder skim milk was a product of Carnation Co. USA. Tween 20 was obtained from ICN Biochemicals Inc. Micro BCA Protein Assay reagent kit and Blocker™ Casein (No. 37528ZZ) (diluted to be 0.1 % with PBS) were obtained from Pierce Chem. Co., Rockford. USA. Cellulofine GCL-1000m was a kind gift of Chisso Inc.

Phosphate buffer salin (PBS) pH 7.2 1 l preparation

$NaH_2PO_4 \cdot 2H_2O$	2.6g
$Na_2HPO_4 \cdot 12H_2O$	0.42g
NaCl	8.18g

Dissolve in double distilled water to be 1 l.

Phosphate buffer salin Tween (PBST) pH 7.2 1 l preparation

$NaH_2PO_4 \cdot 2H_2O$	2.6g
$Na_2HPO_4 \cdot 12H_2O$	0.42g
NaCl	8.18g
Tween 20	500 μl

Dissolve in double distilled water to be 1 l.

2.5.3.2. **Preparation method of blocker-stabilizer-diluent fraction**

15g of skim milk powder (Carnation Co.) was dissolved in 50 ml of PBS (3%) and autoclaved at 120 °C for 10 min and right away carefully open the autoclave and centrifuged for 10 min at maximum speed (7000 rpm) in a clinical centrifuge. The clear supernatant was taken carefully. About 20 %

protein was removed as precipitation by centrifugation determined by Micro BCA Protein Assay. This supernatant is used as blocker-stabilizer-diluent by diluting 10 to 20 times with buffer in EIA.

2.5.4. **Fractionation by gel filterration**

The above fraction was fractionated by Cellulofine GCL-1000m column (1 x 37 cm, 38ml Cellulofine beads was packed). The elution was done by H_2O. Sample 1ml per tube (=32 drops) was collected in 50 fractions by Advantec SF-2120 Fraction Collector at flow rate of 1drop per 2 min 30 sec (Fig. 2-3). The peak was monitored at 280 nm (Fig. 2-4).

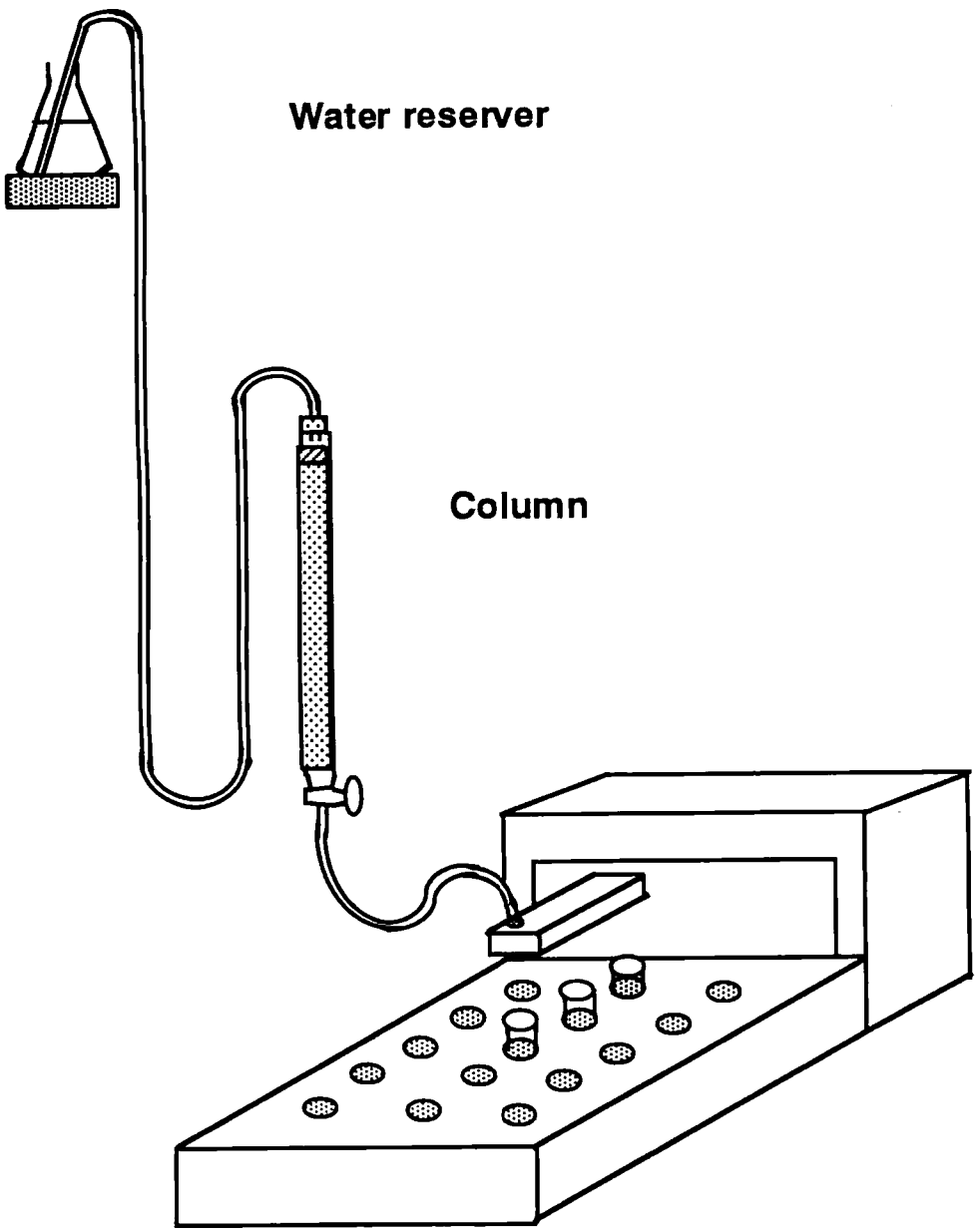


Fig. 2-4. Gel fitration column system

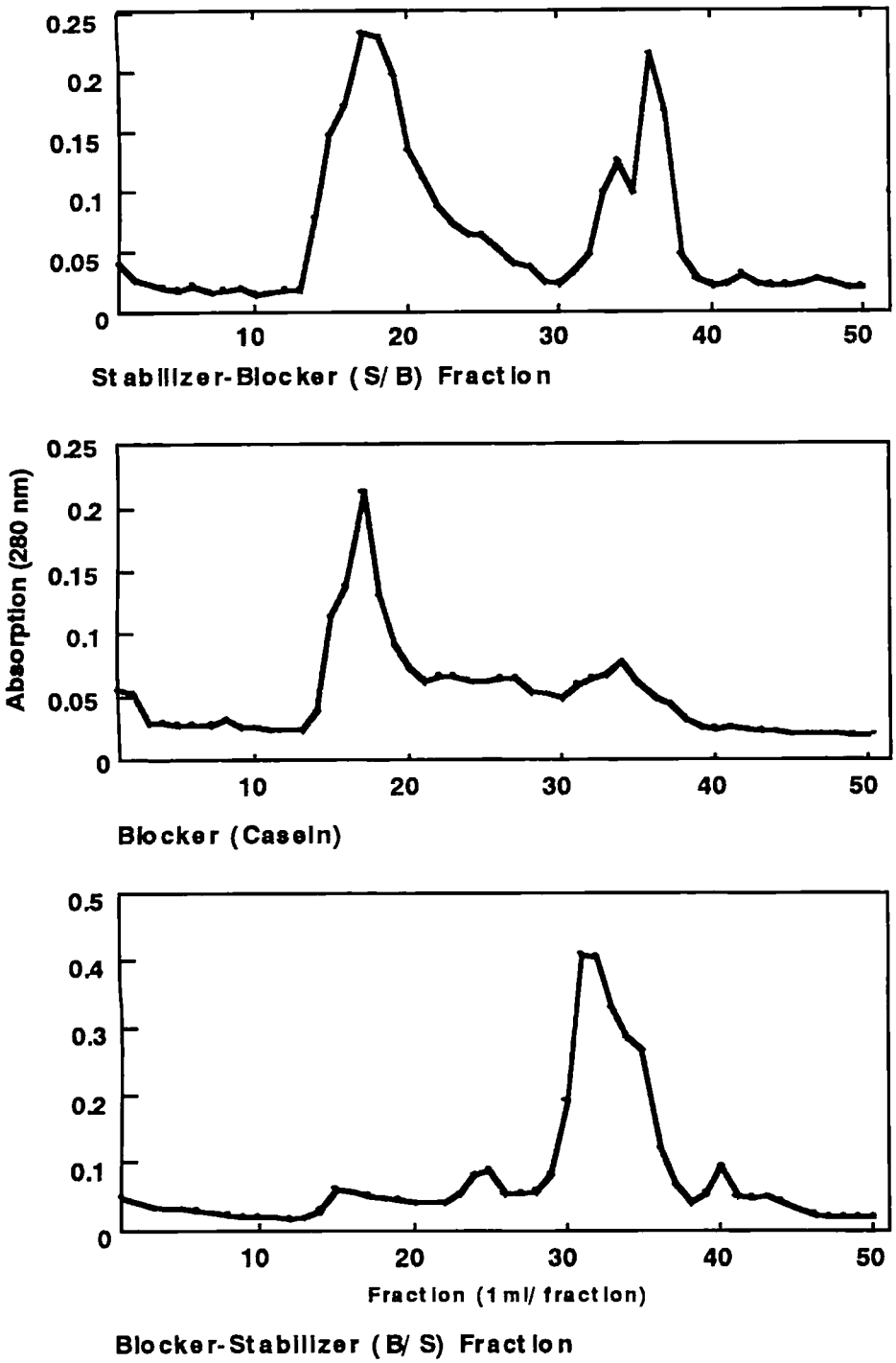


Fig. 2-5. Gel filteration Chromatography of Stabilizer-Blocker

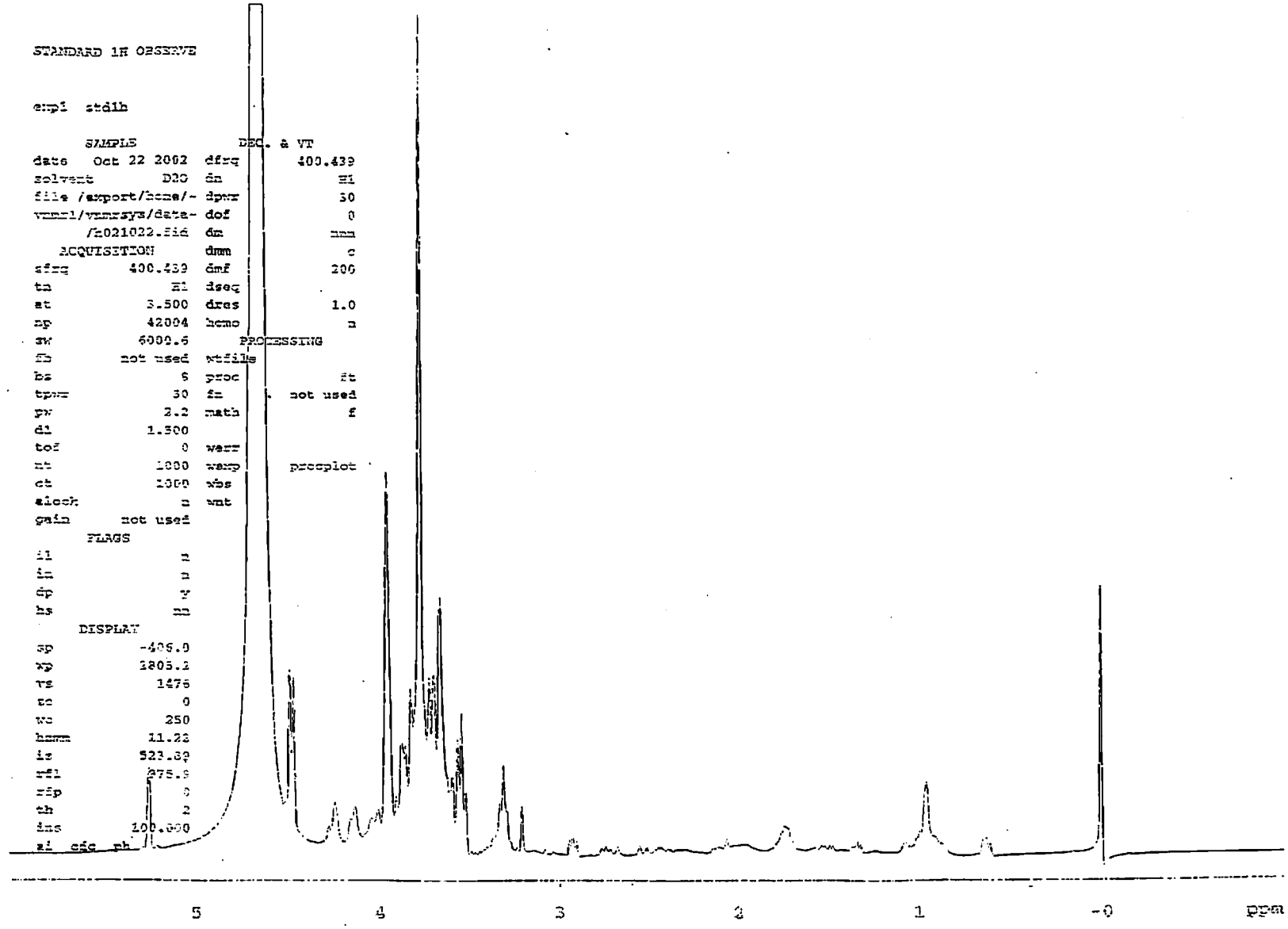


Fig. 2-6. NMR of Blocker-Stabilizer Fraction

From Fig. 2-5, the above fraction (B/S graph) gave two major peaks. The first major peak is casein (casein graph) which had 160,000-200,000 in molecular weight and the second major peak was rather low molecular weight of 500- 1000 which peak came out later than polymyxin B which has molecular weight of 1020. Casein is known for its strong blocking ability. The second peak was subjected to NMR which seems indicates that it contains oligosaccharide as a major portion from Fig. 2-6. The element analysis showed very low content of nitrogen although the sample was not enough high purity.

2.5.5. **Stabilization study of HRP, antibody, HRP conjugate exposed to 50°C**

Assay method to study stabilization and the results

In a 500μl capacity Eppendorf micro centrifuge tube with the lid inner hollow filled with Parafilm to prevent condensation during incubation, 300μl of sample as listed below was placed. The samples were to see whether the above stabilizer can stabilize antibody-enzyme conjugate or enzyme itself or antibody itself when incubated at 50°C.

Samples; (a) CSA-conjugate in PBS, (control)

(b) CSA-conjugate + Casein in PBS

(c) CSA-conjugate + Stabilizer in PBS

(d) Anti *E. coli* 0111 antibody (mouse) in PBS, (control)

(e) Anti *E. coli* 0111 antibody (mouse) + Stabilizer in PBS

(f) HRP in PBS, (control)

(g) HRP + Stabilizer in PBS

Casein and Stabilizer were all in 0.1 % concentration.

For CSA-conjugate (a)~(c) stability assays, 5μl of *Salmonella typhimurium* LPS 1 mg/ml was spotted on a 6x6 mm PEI cloth for each assay. For *E. coli* antibody, (d) and (e), 5μl of *E. coli* 0111 LPS 100μg/ml sample was spotted same way. Free enzyme HRP (g) 1 ng was contained in 300μl sample. Each sample assay was established by adjusting to the incubation time period at 30°C with soluble TMB to have readings of absorbency 0.7 to1.0 at 450 nm taken as

100% activity at 0 time before starting to incubate at 50°C.

Assay method;

For (a) to (e), *Salmonella* LPS or *E. coli* LPS sample 5 μl was spotted on a 6x6 mm PEI-cloth. Incubated for 15 min at room temperature then washed with PBST on a suction filter. Then each (a)~(e) sample was diluted 10 times in PBS to be 50 μl and applied on the respective cloth with LPS on. Incubated for 15 min at room temperature then washed with PBST same way as before. For (d) and (e), one more step to treat same way with second antibody HRP labeled Goat anti-mouse IgM was done. Then each cloth was put into 1 ml soluble TMB solution and incubated in a 30°C water bath shaking vigorously for each specific duration. For (f) and (g) samples, directly 1 μl was withdrawn and added into 1 ml soluble TMB solution and incubated same way as other samples. At the end of reactions, 250 μl of 2N H_2SO_4 was added to each sample to stop reaction and measured the absorbency at 450 nm. These results at 0 time were taken as 100% activities for each sample. Then all sample tube lids were tightly closed and placed in each aluminum block test tube hole (diameter 13 mm) in a block heater (Iwaki Alumibath ALB-221) and incubated at 50°C. The changes of HRP activities were measured according to the method described above by day intervals.

As seen in Fig. 2-7, the stabilizer can stabilize CSA conjugate (c) and HRP (g) for almost one month without losing any activity, while the sample without stabilizer (a) lost activity within 3 days completely. Antibody (e) was also stable without any loss of activity but it is quite stable even without the stabilizer's help (d). On the other hand, casein (b) did not stabilize CSA conjugate at all like buffer itself. HRP itself (f) lost activity very quickly at 50°C without help of the stabilizer. These results indicate that the blocker-stabilizer-diluent fraction has strong stabilizing effect on HRP conjugate, HRP and antibody.

According to FDA's accelerated temperature study for enzyme activity at 50°C, 4 weeks exposure is equivalent to 7 month stability at room temperature exposure (Table 2-1). Thus the HRP CSA conjugate is stable at least stable for 7 month at room temperature storage,

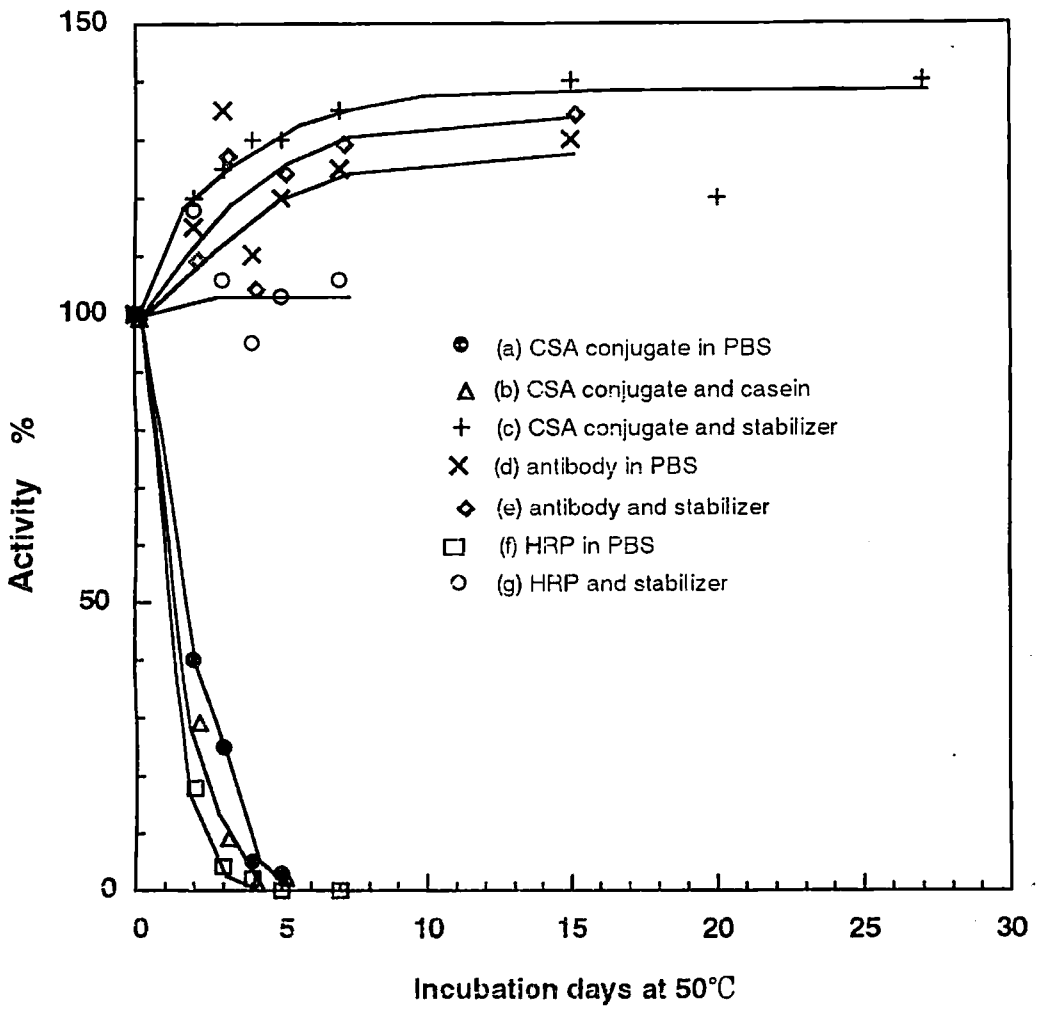


Fig. 2-7. Stabilization study of Stabilizer-blocker-diluent at 50℃

Incubation Time	50°C	37°C
1 week	= 6.1 months at 4°C = 1.7 months at RT	= 2.5 months at 4°C = 0.7 months at RT
2 weeks	= 12.1 months at 4°C = 3.5 months at RT	= 4.9 months at 4°C = 1.4 months at RT
3 weeks	= 18.2 months at 4°C = 5.2 months at RT	= 7.4 months at 4°C = 2.1 months at RT
4 weeks	= 24.3 months at 4°C = 7.0 months at RT	= 9.8 months at 4°C = 2.8 months at RT

Table 2-1. Accelerated Temperature studies for Enzyme Activity (13)

2.6. Summery for Chapter 2

EIA is a simple. specific and sensitive technology for detecting microbial antigens. As the system is using very unstable biological materials. it is desired to have more stable reagents. Cases like TMB or CN which are enzyme substrates but not soluble in water cast problems in EIA. The newly developed preparation make possible such compounds to solubilize in water while keeping it in solid form. Water insoluble TMB in powder form became water soluble preparation made possible to prepare powdered form of TMB reagents. which were very sensitive substrates for HRP and stable under dry, ambient temperature standing at least 7 years. Also the new preparation of blocker-stabilizer-diluent fraction from powder skim milk is simple to prepare and a very useful, unique and powerful blocker-stabilizer and stabilized CSA conjugate for a month incubated at 50°C which is equivalent to stabilize for 7

month at room temperature may contribute to stabilize other enzymes, antibodys and conjugates. The characterization of the blocker-stabilizer is not complete but it may be a oligosaccharide having possible amino acid with molecular weight of 300 to 1000 and rather heat stable compound.

2.7. **References for Chapter 2**

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Chapter 3 **Preparation of polymyxin and PEI-polyester cloth**

3.1. **Matrix support for EIA**

In enzyme immunoassay (EIA), antigen or antibody adsorbed on a solid support made of polystyrene for microwell-based EIA (well-EIA) or on a membrane usually made of nitrocellulose or PVDF for the dot blot method. Hydrophobic macro porous non-woven polyester cloth (SONTARA 8100™) as the solid support, Blais and Yamazaki (1) (2) (3) have developed cloth enzyme immunoassay (CEIA) which used polymyxin B coated-polyester cloth to react preferentially with the Gram-negative bacteria's lipopolysaccharide (LPS) such as *Salmonella.* Further developments and improvements toward commercialization in mind made on p-cloth are presented here. As PEI is also known to react with LPS preferentially (4) (5). PEI-cloth was prepared and applied to use for CEIA. PEI is inexpensive and easily commercially obtainable with many choices of different molecular weights. PEI is adsorbed (by physisorption method) like polymyxin or chemically bonded (by chemisorption method) on polyester cloth.

3.2. **Preparation of polymyxin-cloth**

3.2.1. **Materials**

Non-woven polyester cloth SONTARA™8100 was a gift from DuPont Wilmington. Delaware. Polymyxin B sulfate (7500 U/mg) was obtained from Dumex Co. Belgium. Nile blue A; 12,147-9 and poly(ethylene glycol) (PEG) MW 10,000; 30,902-8 were from Aldrich Chem. Co. Milwaukee, Wisconsin.

3.2.2. **Preparation of washed polyester cloth**

It was necessary to wash polyester cloth because of dirt and oil originated from production and printing, which effected on modifications and consequently to CEIA. Polyester cloth (8 x 5.5 cm) used for modifications was washed with 20% ethanol for few min., then with hot water and cold water.

Here, water indicates double distilled water or deionized then distilled water through out.

3.2.3. **Preparation of polymyxin-coated polyester cloth**

Polymyxin-cloth (p-cloth) was prepared according to Blais and Yamazaki (1) with modifications to overcome the crucial draw back that dry p-cloth does not accept sample. A polyester cloth (5.5 x 8 cm) was wet and blotted then immersed in 4 ml of 5 mg/ml polymyxin B solution. It was sealed in a vessel (a square culture plate of 9.5 x 9.5 x 1.5 cm or plastic baseball card box) with Parafilm and placed in a 40°C incubator over night. The polymyxin solution was recovered by filtration and then the cloth was well washed with water on a suction filter. The polymyxin solution was reused up to 4 times by supplementing with fresh polymyxin solution back to the original concentration, which was monitored at 260 nm by spectrophotometer described below. The quality of p-cloth was monitored by Nile blue color test described below. Finally the blotted cloth was evenly wet with 3ml of 5 % polyethylene glycol (PEG) 10,000 solution on a glass plate and air-dried.

3.2.4. **Nile blue color test to detect polymyxin on cloth**

It is important to have a simple and quick quality control method. Nile blue dye method was developed to detect whether enough polymyxin evenly modified on the cloth. The prepared p-cloth (before application of PEG) was immersed in 0.05% Nile blue solution for 30 min then washed well with water. If active polymyxin is enough on the cloth, the color will show as light pink but otherwise light sky blue will be observed. This method also allows to observe if the cloth is evenly modified.

3.2.5. **Method to reuse polymyxin solution**

For p-cloth preparation, 5 mg/ml polymyxin B solution was used. The standard curve of Nile blue concentration and absorbency at 260 nm was constructed. The absorbency of the 5 mg/ml polymyxin B solution at 260 nm

was 0.327 which was taken as 100% (5 mg/ml). After use of the solution, the lost volume and absorbency were determined. From the standard curve, lost amount of polymyxin was calculated and the equivalent amount of polymyxin B in fresh solution was added and then reused. It is safe to reuse up to 4 times by this method. The resulting p-cloth quality was monitored by Nile blue color test.

3.3. **Preparation of PEI cloth** (6) (7)

3.3.1. **Phenol red method to detect and quantify PEI on cloth**

A 6x6mm size of PEI-cloth was immersed in 50μl of 0.5% Phenol red solution for 30 min at room temperature. Then the unbound dye was completely washed off with water. According to the amount of PEI, light red to deep red color on the cloth was observed. The deeper the red color, the more PEI attached on the cloth. This method is applicable in general to detect amine contained matrix such as DEAE-cloth. Same way 0.5% copper sulfate solution can be used by coloring in blue but is not as sensitive as phenol red method. As minor amount of amine group of PEI contained on cloth was difficult to determine reproducibly by pH back titration method, phenol red method was developed utilizing that phenol red reacts lineally with PEI at low content. The phenol red dyed cloth was placed in a test tube contained 1ml of 0.1N NaOH to extract Phenol red dye. Released Phenol red amount was determined at 559 nm absorbency and quantified from a standard curve of absorbency-amine group content in meq (mM/g-cloth). It was calculated as amine group content in meq (mM/g-cloth) based on an assumption that one molecule of phenol red binds to one amine group of a unit in PEI molecule attached on polyester cloth. This method is applicable for qualitative and quantitative ways and for examining even modification of the cloth.

3.3.2. **Preparation of PEI-coated polyester cloth (PEI-A-cloth); (physisorption method)**

Polyester cloth (5.5x8 cm piece for multiple samples) immersed in 5ml of

5% (w/v) PEI 800 solution (or 6x6 mm piece for individual sample in 50 μl PEI solution) was incubated for 2hr to over night at 40°C. After incubation, the cloth was washed with water on a filter with suction and then air dried. The dried PEI-cloth accepted samples without any further modifications like done on p-cloth.

3.3.3. **Effect of molecular weight of PEI in adsorption on polyester cloth**

PEI of molecular weights 800, 2000, 25000 and 70000 in 5% solution were used for the adsorption on polyester cloth. In 50 μl PEI solution, a 6x6mm polyester cloth was immersed and incubated for various times at 40°C. The level of adsorption was detected by using phenol red color test and the adsorbed PEI on the cloth is expressed as amine group content shown in Fig. 3-1. It shows that very wide ranges of PEI adsorbed on the cloth is available by choosing molecular weight. The adsorption behavior of PEI is molecular weight dependent, and the amount of PEI adsorbed is kinetically governed. As an example after 7 hr incubation, the amine group content of the cloth increased from 0.2 to 2.3 meq/g as PEI molecular weight on the cloths increased from 800 to 70000. This is possibly low molecular weight PEI lay flat to occupy and saturate on fiber surfaces resulting in low content, but larger molecular weight PEI lay flat in part while the remaining portion formed loops and strings which fail to occupy directly on the fiber surface and thus contribute to the higher amine content.

On the other hand, the difficulty in controlling the adsorption of PEI onto cloth at initial stage in case of high molecular weights of 25000 and 70000 was easily overcome by lowering the concentration of PEI solutions to the same amine content dilution as the low molecular weight of desired. In this way it was possible to prepare the same low amine content cloth with any molecular weight of PEI, as long as the reaction solutions had the same concentration in amine group content, rather than molecular weight. It will be explained in the following Chapter that CEIA requires rather low amine content PEI cloth.

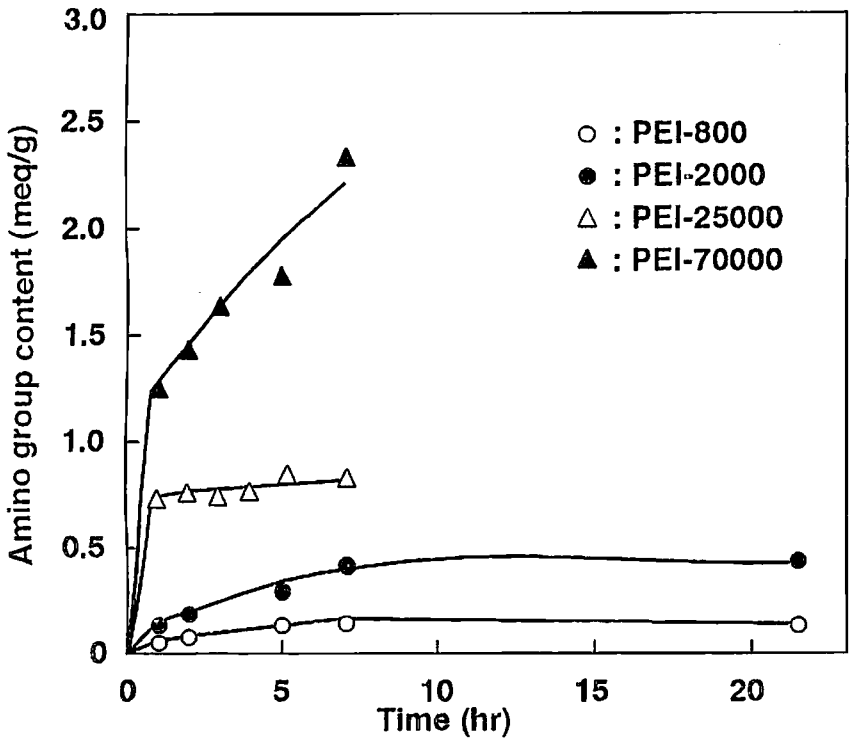

Fig. 3-1. Kinetics of PEI (Mw, 800, 2000, 25000 and 70000) adsorption on polyester cloth detected by the phenol red dye test. Various molecular weights of PEI in 5% aqueous solution were used to coat 6x6 mm polyester cloth by incubating at 40 ^{o}C.

Thus PEI-A-cloths with very wide ranges of amine group content can be prepared by choosing combinations of molecular weight, concentration and incubation time. For example, PEI-A-cloths with equal amine group contents (e.g. calc. 0.13 meq/g) were obtained even when prepared from different molecular weights of PEI by using concentrations (w/v %) of PEI 800 0.5%, PEI 2000 0.2%, PEI 25000 0.016% and PEI 70000 0.0057% incubated for 3 hr at 40°C. The actual amine group contents of those prepared PEI-cloths measured by phenol red method fell between 0.13 to 0.37 meq/g. The variations are possibly due to the reflection of PEI molecular weight distribution determined as polymer.

3.3.4. **Preparation of PEI-covalently bonded polyester cloth (PEI-B-cloth); (chemisorption method)**

PEI can be covalently bonded on polyester cloth by a reaction of acylation of amine (of PEI) by ester (of polyester cloth) under anhydrous condition. Water free PEI in molecular weights of 800 and 25000 were diluted to 70%(v/v) with absolute alcohol to reduce viscosity. A dried 5.5x8cm polyester cloth on a glass plate was spread evenly with 4 ml of 70% PEI solution in absolute ethanol by using spatula. Then it was heated on a dry bath at 110°C for 2 min to 1 hr in a fume hood according to the modifications desired. The reaction was stopped by immersing the cloth in water for few hours to remove non-reacted PEI then further washings by decanting several times and washed with water on a macroporous filter by suction and followed by air drying. Fig. 3-2 shows the kinetics of PEI modification by the amide forming reaction. PEI 25000 appears to incorporate faster and more than PEI 800, but this was due to the bigger molecular size, as was the case in PEI-A-cloth preparation. Through this method, PEI 800 can be introduced more than that of equivalent PEI-A-cloth, because of the higher concentration been used and chemical incorporation.

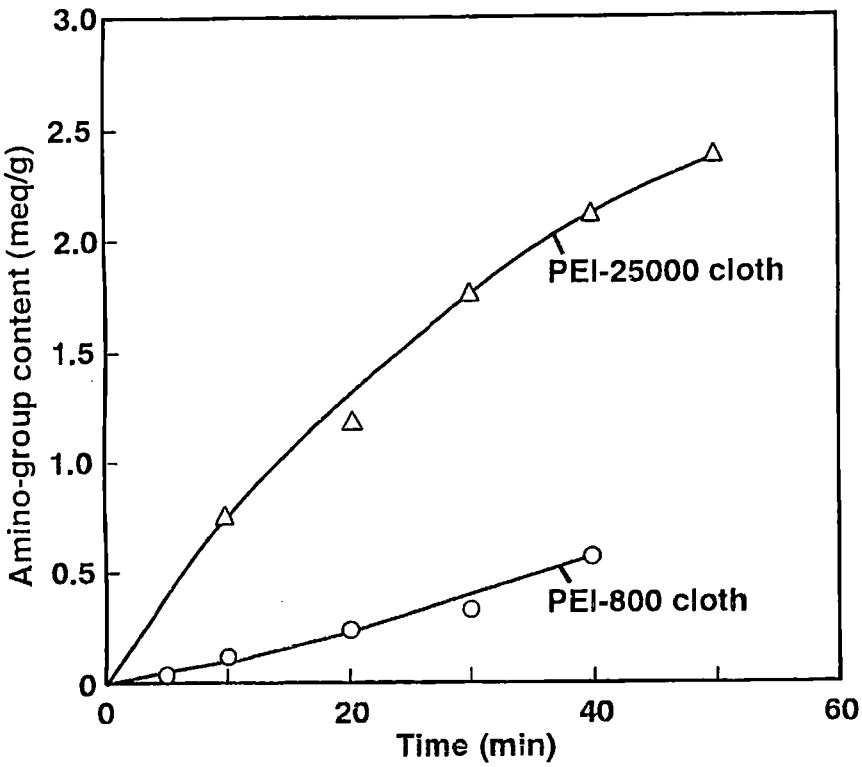

Fig. 3-2. Kinetics of PEI 800 and 25000 chemisorptions to polyester cloth with time.
70%(v/v) PEI 800 and 25000 solutions in absolute ethanol solutions were spread evenly over polyester cloth and heated at 110 ^{o}C for various lengths of time and the kinetics monitored by the phenol red method.

PEI cloth dyed with phenol red or $CuSO_4$ in Fig. 3-3. shows the progress of the reaction at 110°C.

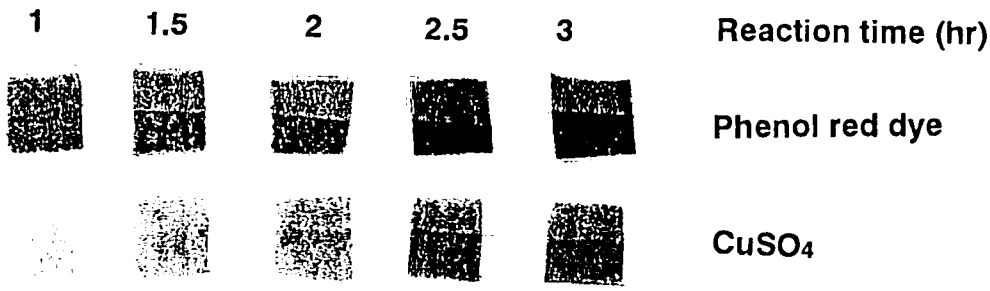

Fig. 3-3. PEI 800 chemisorption reaction with polyester cloth at 110°C

It was possible to prepare wide ranges of PEI-B-cloth as with PEI-A-cloth, but the reaction must be stopped before the limit at which cloth weakens occurs, because excess reaction time causes fragmentation of the fiber as the reaction occurs by cutting polyester's fiber. Fig. 3-4 shows some possible chemical structures of PEI-polyester resulted from primary amine and ester by amide exchange reactions. The fragmentation of the fiber could be proof that the acylation reaction occurred, but the amide peak was not confirmed by FT-IR possibly due to such a small number of bonds being formed compared to absorption of polyester. This reaction occurred with monomeric amine compound and excess reaction caused fragmentation of the fiber as shown in Fig. 3-5.

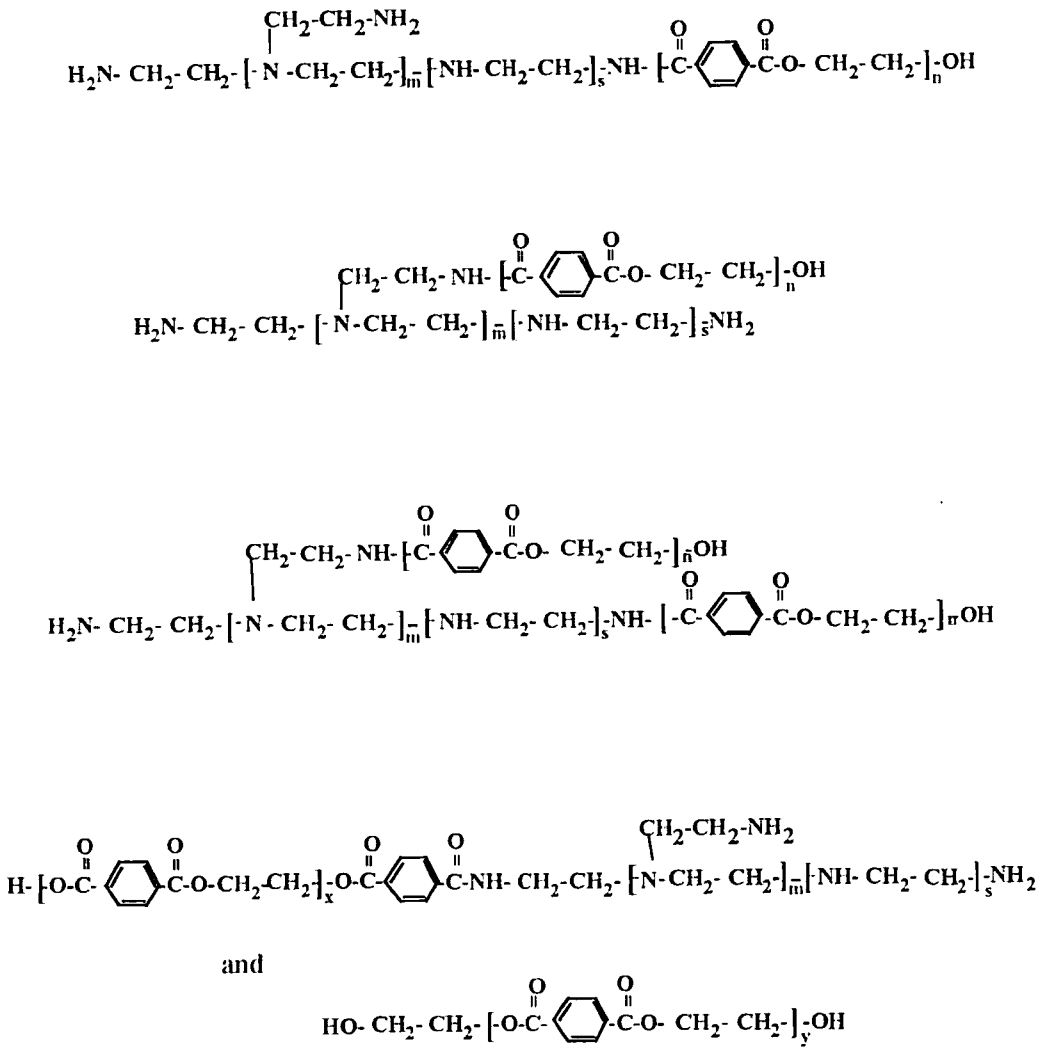

Fig. 3-4. Some possible structures of PEI-polyester cloth after chemisorption reaction

3.3.5. **Preparation of 3,3' imino bis(propylamine)-modified polyester cloth (IBPA-cloth) (chemisorption method)**

A dried 5.5x8 cm non-woven polyester cloth was placed on a glass plate and evenly spread with 4 ml of 3,3'-imino bis(propylamine) and heated at 80°C for 2 to 40 min on a dry bath in fumehood. Washing was done in the same way as

PEI-modified cloth. Fig 3-5 shows the progress of the modification reaction monitored by phenol red dye method and eventually destruction of polyester fiber occurred. The phenol red dye coloration and deterioration of cloth may indicate the reaction of IBPA incorporation into polyester.

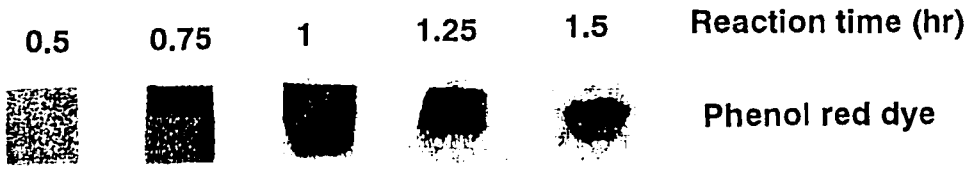

Fig. 3-5. IBPA chemisorption reaction with polyester cloth at 80°C.

3.4. **Summery for Chapter 3**

P-cloth was improved by coating with PEG. This extra procedure made possible for dry p-cloth to accept samples. The adsorption of polymyxin onto polyester cloth was limited to amine content of 0.05 meq/g-cloth, which may explain the difficulty of absorbing sample or water from dry state. The value did not change after many attempts to enhance the adsorption, for example by higher ionic strength, longer incuvation, higher temperature etc. Either coated or chemically bonded, PEI-cloth is easy to prepare as polymyxin-cloth and PEI is about 100 times less expensive than polymyxin. As polymyxin-cloth was stable enough to be used after 10 years stored at room temperature, the PEI-cloth could be as stable as polymyxin-cloth (so far 7 years). PEI can be introduced onto polyester cloth in very wide ranges of amine content and certain specified amount by using PEI of any molecular weight through adsorption. On the other hand, PEI in anhydrous condition reacts with polyester and introduced by ester exchange reaction. A monomeric amine IBPA was much more reactive than PEI and fragmentation of cloth occurred much faster at lower temperature than PEI. This is a very simple and useful method to introduce amine group on polyester cloth in wide ranges of amine group content.

3.5. **References for Chapter 3**

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Chapter 4. **Application of PEI-cloth for cloth enzyme immunoassay**

4.1. **PEI-CEIA**

Enzyme immunoassay (EIA) is a rapid and reliable method to detect contaminated pathogens, organic materials, toxins etc as antigens. P-CEIA is a very effective antigen detecting method to monitor *Salmonella* and other gram negative bacteria (1) (2) (3) because of its large cloth surface area, efficient capture of antigen and washing. As PEI is a cationic polymer and reacts with LPS preferentially (4) (5). PEI-CEIA was compared with p-CEIA (6) (7) and further applied to detect very minor amount of LPS contaminated in large volume sample by concentrating (8) (9).

4.1.1. **Chemicals and immunoreagent**

Salmonella typhimurium LPS (No. L-6511) was obtained from Sigma Chemical Co. Horse radish peroxidase (HRP) labeled Goat anti-*Salmonella* common structural antigen (CSA-1) (No. 04-91-99) was a product of KPL, Inc., which was reconstituted in 50% glycerol to be 0.1 mg/ml and stored at –20°C. It was diluted 2000 times in PBS contained 0.1% blocker for use. *E. coli* strain 0111:B4 LPS (No.3922-25-0) was from Difco laboratories. Mouse anti-*E. coli* 0111 (mono, No. MON2011) was obtained from SANBIO BV. HRP labeled Goat anti-mouse IgM (poly, No.SAB-110) was a product of StressGen Biotechnologies Corp. Soluble and Insoluble TMB reagents in powder form were gifts from Ricoh Kyosan Inc. Tokyo, and dissolved 35 mg in 1ml water before use.

4.1.2. **Preparation of LPS sample**

Salmonella and *E. coli* LPS (both in 10 mg/ml stock solution) were diluted in PBS contained sodium cholate 5.5 mg/ml and brilliant green 5μg/ml to various concentrations by 10 times in stepwise and heated at 100°C for 10 min. Sample of 5μl was spotted on the cloth for CEIA. Cholate works to prevent LPS from forming micelle and the dye is to visualize the spotted samples on the

cloth.

4.1.3. **Cloth enzyme immunoassay (CEIA) method**

The assay method was followed after that of Blais and Yamazaki (1). On a dry 6x6mm PEI- or p-cloth, 5μl of LPS sample was applied and incubated for 15 min at room temperature. The cloth was washed with 1ml PBST on a macroporous filter under suction and blotted. For *Salmonella* sample, 50μl of the CSA-1 HRP conjugate (the stock solution of 0.1mg/ml in 50% glycerol solution was diluted 2000 times with PBS contained 0.1 % blocker-stabilizer) was applied and incubated for 15 min at room temperature. It was washed same way as before (Fig. 4-1A). In case of *E coli* 0111 LPS sample, the cloth was incubated with 50μl of 20 times diluted mouse anti-*E. coli* 0111 (first antibody) in 0.1 % blocker-stabilizer in PBS for 15 min then washed with PBST same way as before. It was further treated with 50μl of anti-mouse IgM-HRP conjugate (second antibody), which was diluted 2000 times in PBS contained 0.1 % blocker-stabilizer, for 15 min and then washed with PBST (Fig. 4-1B). Such a way prepared cloth was placed in a 16 x 100 mm test tube contained 1 ml soluble TMB solution and incubated at 30°C in water bath with shaking for 5-60 min. At the end of reaction, 250μl of 2N H_2SO_4 was added and its absorption at 450 nm was determined. The actual operations are shown in Fig. 4-2.

4.1.4. **Dot blot assay**

P- or PEI-cloth in the size of 5.5x8 cm was spotted with 5μl samples at 1 cm interval or within printed circles. The method was followed exactly same way as CEIA up to the final stage of TMB reagent application. At final stage, 4ml of insoluble TMB reagent (insoluble TMB powder reagent 35 mg dissolved in 1 ml water) was applied and let HRP react for 5-60 min at room temperature. The sheet was then washed with water over suction filter and examined for blue spots against white background. These sheets could be stored as semi-permanent records.

4.2. Effect of molecular weight of PEI in adsorption on polyester cloth and its suitability for CEIA

Molecular weights of PEI 800, 2000, 25000 and 70000 in 5% solution were used for the adsorption on polyester cloth. In 50μl PEI solution, a 6x6mm polyester cloth was immersed and incubated for various length of time at 40°C. Level of adsorption was detected by using phenol red method as shown in Fig. 3-1. It showed that PEI adsorbed on the cloth in very wide ranges was available by choosing a molecular weight. Fig. 4-3a shows that which level of PEI on the cloth is suitable for CEIA was monitored by Dot blot assay. For CEIA, molecular weight of 800 was found to give clear and strong blue spots with white background. While other PEI-cloth with higher molecular weights had strong blue background or even the spot could not be identified. This is possibly because the abundant amino groups of PEI bound the enzyme-antibody conjugate nonspecifically. These results indicate that the amine group content 0.1~0.35 meq/g as PEI on cloth is suitable for CEIA use because of their clear tight spot with low back ground.

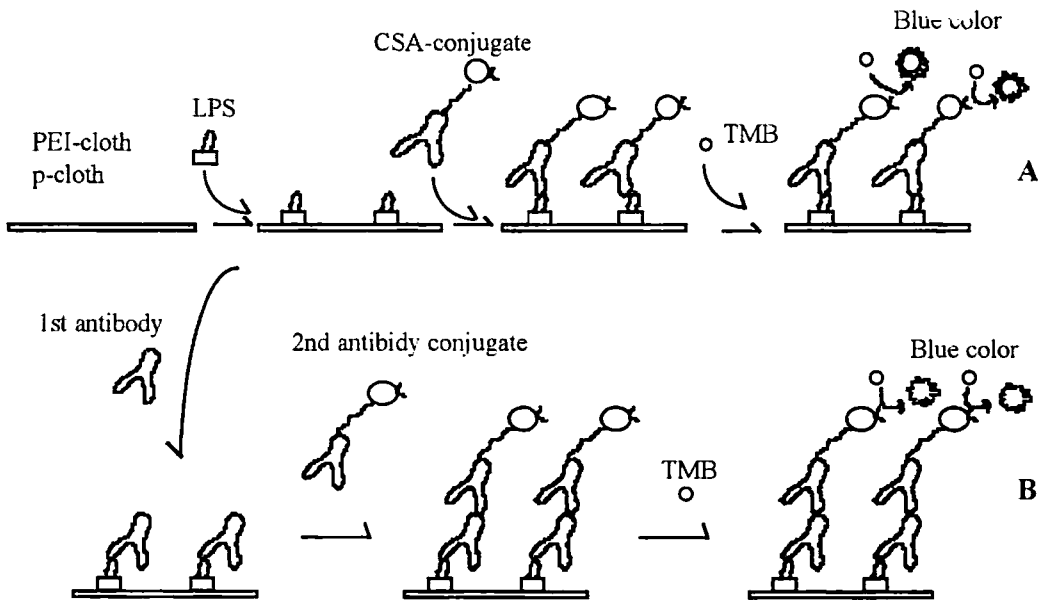
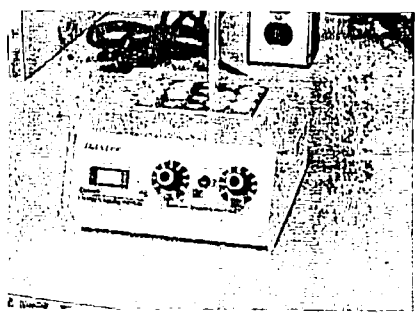


Fig. 4-1 A & B, Schematic presentation of two examples of CEIA

Fig. 4-2 PEI-cloth enzyme immunoassay method to detect Gram-negative bacteria

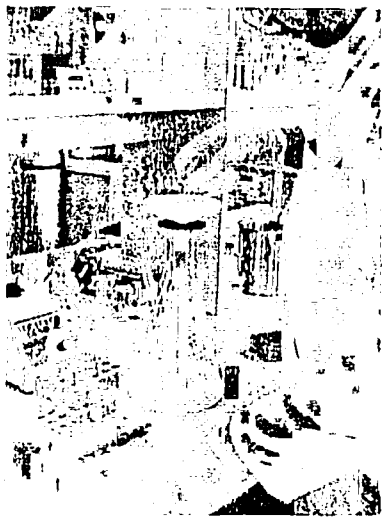
1. Add 1/10 volume of LPS Extractio n Buffer in cell Sample to be tested.

2. Heat the sample at 100℃ for 10 min and then cool to room temperature.

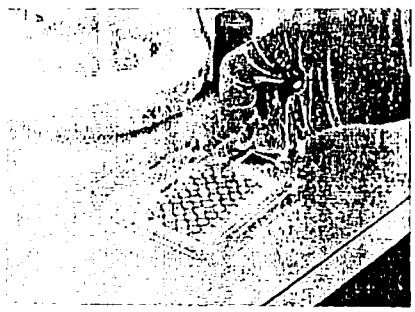


3. Spot 5μl of the heat treated samples on a dry PEI-cloth and incubate for 15 min at room temperature.

4. Wash the cloth with 50 ml PBST on a macroporous filter under suction.

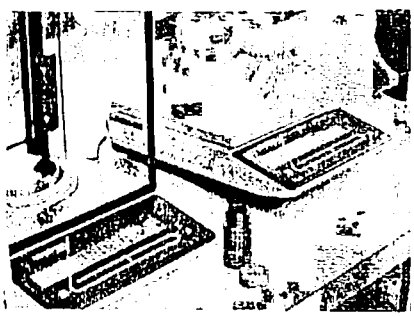


5. Prepare the specific HRP-antibody conjugate diluting in a buffer containing 1/10 vol.of conjugate stabilizer.

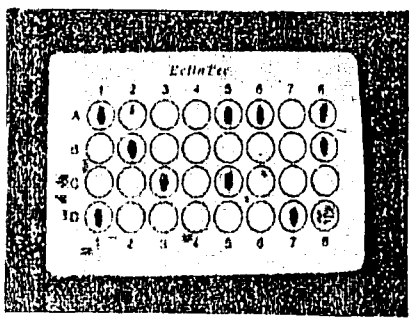


6. Saturate the cloth with 5 ml conjugate solution and incubated for 15 min.

7. Wash as described in 4.



8. Prepare 5 ml TMB reagent (dissolve powder Insoluble TMB reagent 35mg/ml in water) and apply quickly and evenly over the cloth



9. Read the results.

4.3. **Effect of PEI concentration in adsorption onto polyester cloth**

Effect of different concentrations of PEI (Mw 800) for coating polyester cloth in capturing *Salmonella* LPS was studied by using CEIA. A 6x6 mm size of polyester cloth each was placed in 50μl of various concentrations of PEI (1-10 mg/ml) and incubated at 40°C for 16 hr then washed with water and dried. To the dried cloth, 5μl of *Salmonella* LPS (1μg/ ml) sample was applied and assayed according to CEIA method. The last enzyme reaction time for shaking at 30°C was 30 min. The Fig. 4-4 shows that the concentration of 5mg/ml was enough to give maximum CEIA signal and the negative controls were consistently low at all PEI concentrations tested. Thus subsequent experiments were performed with PEI-cloth prepared in 5% PEI 800 concentration unless specified.

4.4. **PEI-covalently bonded polyester cloth and its feasibility for CEIA**

It was found in Fig. 3-2 by CEIA that cloth with PEI 800 reacted for 2-5 min was most suitable and the dot blot assay as seen in Fig. 4-3b. shows by its low back ground and clear spot. However the highly incorporated PEI-cloths with PEI 800 reacted longer than 20 min and PEI 25000 longer than 5 min were inferior for CEIA use because the excess of PEI caused higher background due to the nonspecific bindings of the enzyme-antibody conjugate in spite of blocker presence in high amounts. Thus together with the study in section 4.2., the PEI-cloths with amine contents of 0.1~0.35 meq/g gave consistently clear spot with low background regardless of adsorbing or chemically bonding preparation or by which molecular weight of PEI used. However, a PEI-cloth with very high adsorption capacity could be still useful for other purposes such as removing unwanted LPS or DNA from fluid for therapeutic medicine or chelating heavy metals from industrial wastes etc.

4.5. **Blocker's effect on CEIA**

The blocking effect of the stabilizer-blocker fraction was studied. To PEI-cloths (6 x 6 mm) which were prepared with PEI 800 by heating at 110°C for 10,

20, 30, 40 min, various concentrations of the blocker 0.1~0.9 % without containing LPS were applied. Then CEIA was carried out with CSA conjugate to study the nonspecific adsorption of the conjugate on the PEI-cloths. The absorptions at 450 nm were measured to see the blocking effect as background absorbency (Table 4-1).

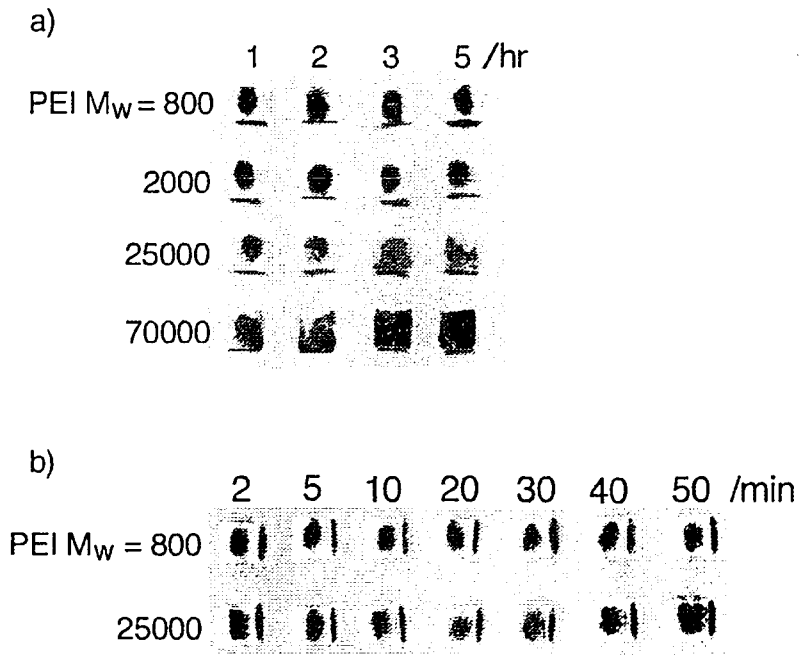


Fig 4-3a and –3b. Dot blot assay response to Salmonella LPS (10 mg/ml), on PEI-A-cloth (a). PEI physisorbed cloth, and PEI-B-cloth (b). PEI chemisorbed cloth, prepared from different molecular weights of PEI reacted for various lengths of time. PEI-A-cloths of PEI 800 and 2000 up to 3 hr reaction, and PEI-B-cloths of PEI 800 were usable for CEIA.

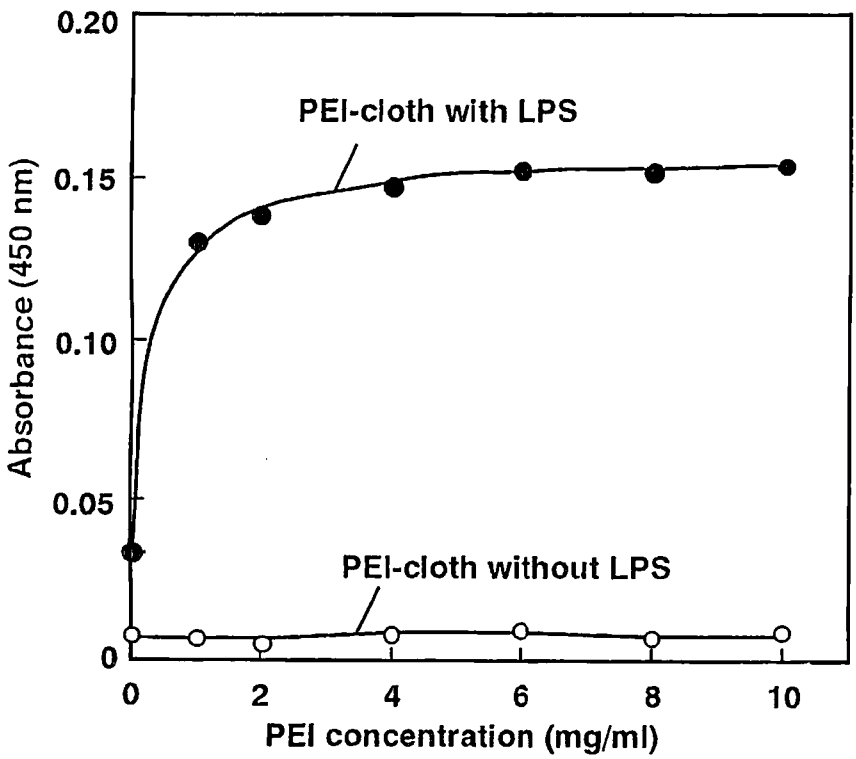


Fig. 4-4. Effect of PEI (Mw 800) concentrations for adsorbing on polyester cloth. A 6x6 mm size of polyester cloth placed in 50 μl of various concentrations of PEI (1-10 mg/ml) solution and incubated at 40 oC for 16 hr. The PEI adsorbed on the cloth was monitored by CEIA. To the dried cloth, 5μl of *Salmonella* LPS sample (1μg/ ml) was applied and the captured LPS was detected by CEIA.

PEI-cloth Reacted Time (min)	Absorbency at 450 nm								
	Blocker Concentration (%)								
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
10	0.019	0.024							
20		0.024	0.022						
30	0.101	0.096	0.048	0.036	0.058	0.058	0.061	0.061	0.061
40	0.109	0.057	0.042	0.032	0.055	0.054	0.061	0.055	0.061

Table 4-1. Effect of blocker concentration on PEI-CEIA using cloths with different amount of PEI on.

As seen in Table 4-1, the higher contents of PEI on the cloth (longer reaction time) gave the higher background absorbency and no improvement in blocking effect from certain point (in longer reaction than 30min) and even by increasing the blocker concentration from 0.4 to 0.9 %.

Using various PEI 800 incorporated cloths, the effect of blocker concentrations on LPS detection limit was tested by dot blot assay (Fig. 4-5A). The dot blot assays showed that blocker's concentration effected on the results and lower blocker concentration cases was detectable at maximum 100 ng/ml level but some back ground color was still observed but on the other hand, with higher concentrations (Fig. 4-5B) was possible to detect up to 100 ng/ml and especially the 10 min reacted PEI cloth with 0.1 % blocker detected 10 ng/ml as a faint spot.

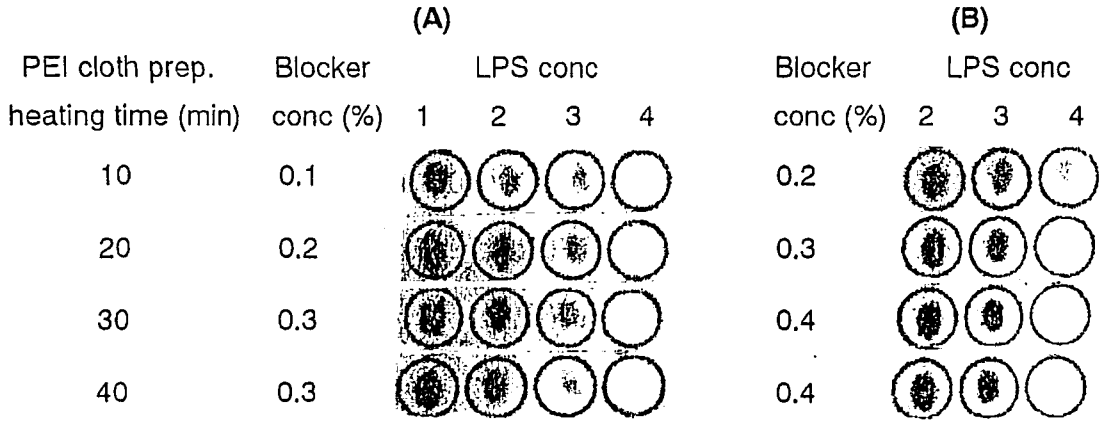


Fig. 4-5A and 4-5B, Blocker's concentration effect on dot blot assay baclground
Salmonella LPS conc. 1; $1x10^4$, 2; $1x10^3$, 3; $1x10^2$, 4; $1x10^1$ ng/ml

Based on this results, PEI cloths prepared by reacting for 5, 10 and 15 minutes were tested with 0.1 % blocker to see the improvement.

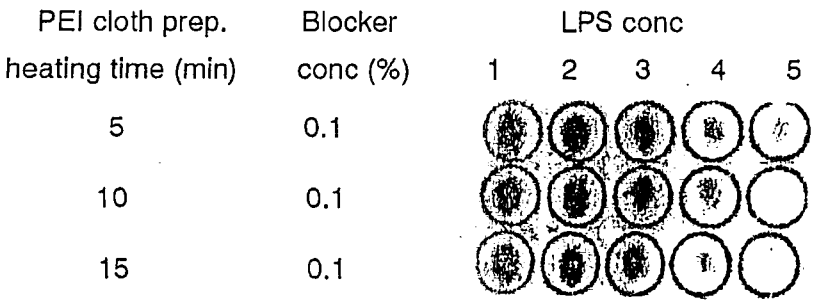


Fig. 4-6, Maximizing assay conditions of PEI cloth preparation and Blocker.
Salmonella LPS conc. 1; $1x10^4$, 2; $1x10^3$, 3; $1x10^2$, 4; $1x10^1$, 5, $1x\ 10^0$ ng/ml

The dot blot assay showed that 10 and 15 min PEI cloth detected 10ng/ml LPS and 5 min cloth was possible to detect 1 ng/ml LPS as seen in Fig. 4-6.

4.6. **Kinetics of LPS captured by PEI-cloth**

The kinetics of *Salmonella* LPS captured on PEI-cloth was detected by CEIA. Fig. 4-7 shows that PEI-cloth can capture LPS completely within 2 min. while polymyxin-cloth takes more than 15 min. More detailed study with PEI-cloth showed that 85% of LPS was captured in 5 sec. and near completion in 10 sec., while polymyxin-cloth was 33% in 5 sec. and near completion in 15 min. The results show the stronger interactions between PEI and LPS than polymyxin-cloth.

4.7. **CEIA response to various concentrations of *Salmonella* LPS using PEI- and p-cloth**

Using 6x6mm of PEI- and p-cloth, 5μl of LPS samples of different concentrations were spotted and the CEIA was carried out as described previously. The final incubation at 30°C with enzyme was 5 min. The comparison in sensitivity of PEI-CEIA and p-CEIA is shown in Fig. 4-8. When *E. coli* LPS was used and the CEIA was carried out as described previously, almost identical with *Salmonella* LPS result shown in Fig. 4-8 was obtained (result not shown). Both cloths gave same sensitivity with detection limit of LPS concentration 100 ng/ml, which corresponds to 1.6×10^6 cells when cell LPS content factor is 0.034, and the weight of a cell is 2×10^{-12} g/cell. Dot blot method gave the detection limit of 10 ng/ml occasionally 1ng/ml for PEI-cloth and on the other hand, 100 ng/ml for p-cloth as seen in Fig. 4-9. These results indicate that PEI-cloth is usable for other Gram-negative bacteria LPS adsorbing media for CEIA.

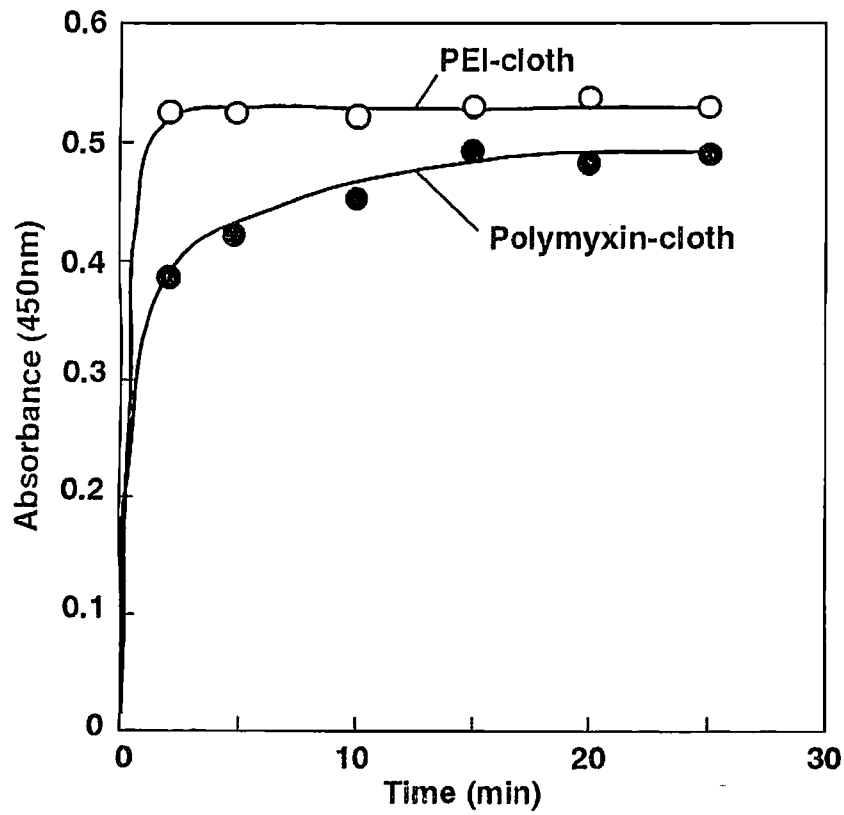


Fig. 4-7. Kinetics of *Salmonella* LPS captured by polymixin-cloth and PEI-B-cloth (PEI Mw 800 reacted for 5 min at 110 oC). 5μl of LPS sample (10 μg/ml) was spotted on 6x6 mm of PEI- and polymyxin-cloth and the captured LPS was followed by CEIA.

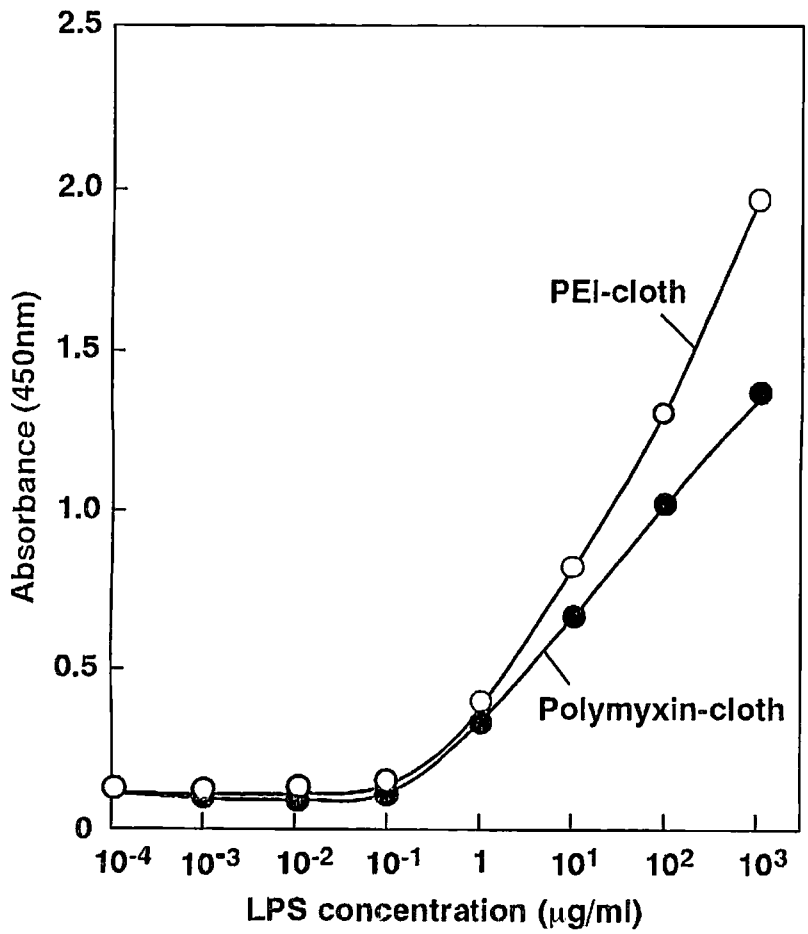


Fig. 4-8. CEIA response to various concentrations of Salmonella LPS captured by PEI-cloth and polymyxin-cloth. PEI (Mw800) cloth reacted for 5 min at 110 oC was used. Using 6x6mm of PEI- and Polymyxin-cloth. 5μl of LPS samples in different concentrations were spotted and CEIA was carried out.

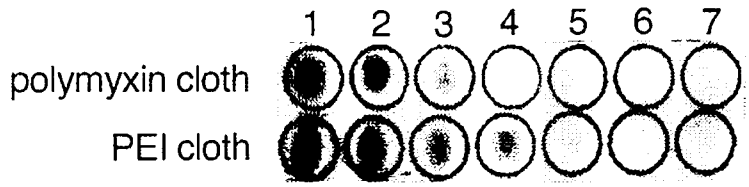


Fig. 4-9. Dot blot assay response to various concentrations of Salmonella LPS on PEI- and polymyxin-cloth. Salmonella LPS concentrations were 1: 100 mg/ml, 2: 10 mg/ml, 3: 1mg/ml, 4: 100ng/ml, 5: 10ng/ml, 6; 1ng/ml, 7: 100pg/ml. Salmonella LPS detection limit of polymyxin-CEIA was 100ng/ml, on the other hand PEI-CEIA was 10ng/ml.

4.8. **Specificity of PEI-CEIA and p-CEIA**

Whether the lipid A region of LPS is responsible for specific adsorption to p-cloth was examined (10). It was found that the lipid A region is necessary for the capture of the LPS antigen by p-cloth. Its two model hydrophobic compounds, deoxycholate and caprylic acid, did not compete for binding site with the lipid A region, thus the binding is possibly not a non-specific hydrophobic interaction. This could suggest that ionic bindings between amino groups of polymyxin and phosphate groups of the lipid A region may also be involved in besides the hydrophobic interaction. As cholate (5.5mg/ml contained in all LPS samples) gave no interfering effect in PEI-CEIA, it may have very similar binding as with p-cloth.

Since PEI-cloth showed even higher *Salmonella* LPS binding capacity than p-cloth, the presence of other Gram-negative bacteria LPS in the samples may compete with each other for binding to PEI-cloth but it should be possible to

detect *Salmonella* LPS. As seen in Table 4-2, when the *Salmonella* LPS detecting PEI-CEIA method was applied on a sample of *Salmonella* LPS contained *E. coli* LPS in a 100 fold excess, it gave same strong signal as the sample of *Salmonella* LPS only, while that of *E. coli* LPS only gave a negligible signal. As an example, a mixture of *E. coli* LPS 100 μg/ml and *Salmonella* LPS 1 μg/ml gave a CEIA signal of 0.143 at 450 nm, while *E. coli* LPS in 100 μg/ml gave 0.029, and *Salmonella* LPS in 1 μg/ml gave 0.121 by using *Salmonella* LPS detecting CEIA. This result shows the specificity of PEI-CEIA, in that it is able to confirm the target bacteria's LPS when other bacteria's LPS are present in the sample.

***Salmonella* LPS (*S*)**		***Salmonella* LPS (*S*) + *E. coli* LPS (*E*)**		***E. coli* LPS (*E*)**	
Sample conc. ratio	A 450	Sample conc. ratio	A 450	Sample conc. ratio	A 450
***S*:*E*=100:0**	**0.551**	***S*:*E*=100:1**	**0.757**	***S*:*E*=0:1**	**0.023**
***S*:*E*=1:0**	**0.121**	***S*:*E*=1:100**	**0.143**	***S*:*E*=0:100**	**0.029**

Table 4-2. Effect of E. coli LPS presence in *Salmonella* LPS sample on PEI-CEIA using anti-*Salmonella* antibody-enzyme conjugate.
On 6x6 mm PEI-cloth (Mw 800), by spotting 5μl samples of among *Salmonella* LPS 1μg/ml, 100μg/ml and *E. coli* LPS 1μg/ml, 100μg/ml in combinations sited as above in concentration ratio and the CEIA was carried out according to Materials and Methods.

4.9. Development of Rapid PEI-CEIA

4.9.1. Background for Rapid PEI-CEIA

It is important to increase the sensitivity of assay so that the prior enrichment step for the target bacteria can be shortened and the results obtained more quickly. For example, a polyester cloth was able to adsorb antibody which in turn captured and concentrated antigens, such as lipopolysaccharides (LPS), in dilution in large volumes, by utilizing its macroporosity, thus enhancing the sensitivity (11). However antibodies have stability problems, are non-uniform quality by lot, and are expensive. Thus polymyxin, which binds LPS preferentially through its lipid A portion and more stable than antibody, was coated onto the hydrophobic polyester cloth (12). Resulting p-cloth was used to capture and concentrate LPS antigen diluted in large volume. Then, the captured antigen was detected by LPS specific antibody-enzyme conjugate (13). We found that PEI polyester cloth (PEI-cloth) was faster in capturing LPS than p-cloth, together with other advantages such as ease of sample handling, better sensitivity, higher LPS adsorbing capacity and reduced cost. PEI-cloth used as a media to concentrate the target LPS diluted in large volumes increase the sensitivity and detectability of *Salmonella*.

4.9.2. **Preparation of PEI-cloth for the rapid LPS detecting method**

A 5.5 x 8 cm dry polyester cloth on a glass plate was soaked evenly with 4 ml of 70%(v/v) PEI 800 solution in absolute ethanol and heated on a dry bath at 110°C for 5min. The reaction was stopped by immersing the cloth in water for a few hours, followed by several decant washings with water and then over a filter with suction. The PEI-cloth was cut to 6 mm diameter circle to fit in a Swinnex cartridge.

4.9.3. **PEI-CEIA Method (A static phase method)**

A 5μl LPS sample spotted on a 6 x 6 mm PEI-cloth (and washed with PBST after 20 min of standing) or LPS captured on the PEI-cloth disk prepared as above were incubated with CSA-1 conjugate containing 0.1 % blocking/stabilizing/diluent for 20 min at room temp, then washed with PBST. The cloth was transferred into a 16 x 100 mm test tube containing 1 ml of

soluble TMB reagent and shaken vigorously for 1~10 min at 30°C. The HRP reaction was stopped by adding 250μl of 2N H_2SO_4 and the absorbency was measured at 450 nm. For the dot blot assay, insoluble TMB reagent was applied on the cloth instead and the HRP reaction was stopped by washing with water. CEIA with p-cloth was done in the same way as PEI-CEIA.

4.9.4. **Comparison of the binding of LPS on PEI-cloth and p-cloth**

Above study in Fig. 4-7 showed that PEI-cloth captured LPS faster than p-cloth. The quick adsorbing LPS by PEI-cloth was applied for concentrating dilute LPS in large volume samples. Fig. 4-10 shows a comparison of LPS capture by PEI-cloth and p-cloth to study in more detail about the initial stage of LPS capture to Fig. 4-7. PEI-cloth captured LPS about 67% in 1 s and 85% in 5 s and reached saturation in 5 min (100%). In contrast, p-cloth captured LPS about 27% in 1 s and took about 15 min to reach its saturation (100%). Thus PEI-cloth was found to be more than 2 times faster in capturing LPS than p-cloth.

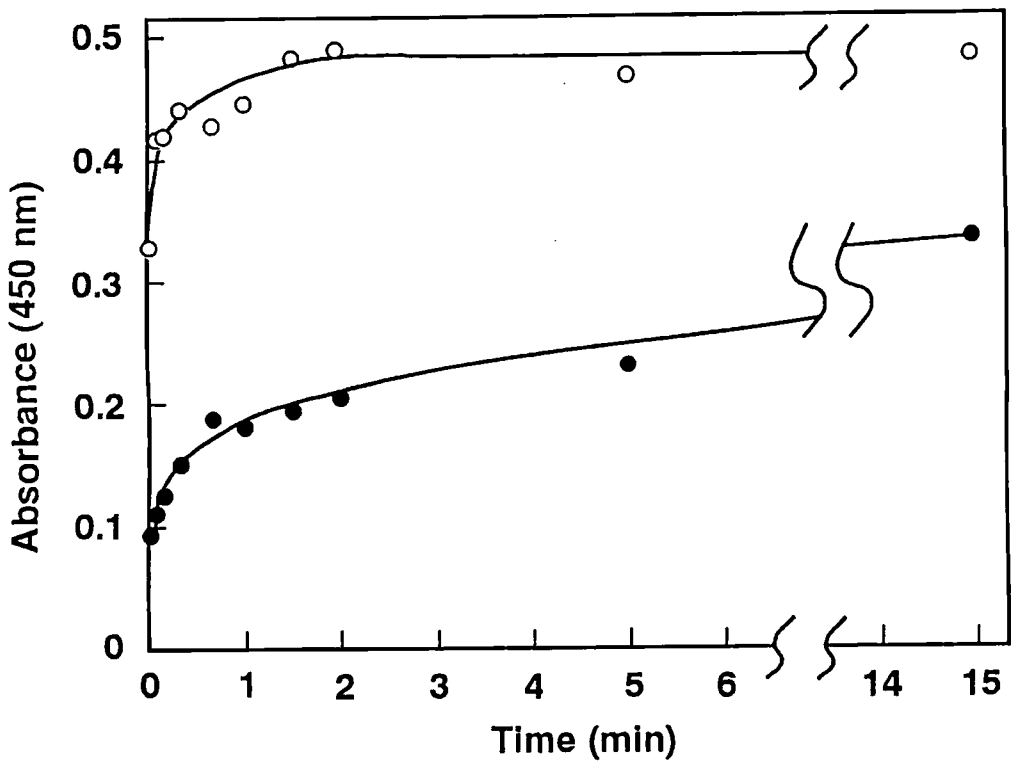


Fig. 4-10. Comparison of LPS capture by PEI-cloth and by p-cloth in static phase.
PEI-cloth captured LPS about 67% in 1 s and 85% in 5 s and reached saturation in 5 min (100%). In contrast, p-cloth captured LPS about 27% in 1 s and took about 15 min to reach its saturation (100%). Thus PEI-cloth was more than 2 times faster in capturing LPS than p-cloth.

4.9.5.1. **Method for concentrating dilute LPS in large volumes by PEI-cloth set in Swinnex-13™ (A mobile phase method)**

A disk-shaped PEI-cloth 6mm in diameter was placed on the filter side of a Swinnex-13™ and covered with a 2 mm thick silicon ring with a 4.5 mm inner

and a 12 mm outer diameter, and was set in the cartridge. Two 10 ml syringes were connected at both ends (one side joined with a mini dual female connector) and a 1 or 10 ml LPS sample in a syringe was passed through the PEI-cloth 7 times, back and forth, at 45 s per 1 ml. A peristaltic pump connected to the cartridge could replace syringes in order to circulate larger volume samples. The cloth was taken out of the cartridge and allowed to stand for 20 min at room temp, then washed with PBST on a filter with suction.

4.9.5.2. **Binding LPS on PEI-cloth in the mobile phase**

The above results in Fig. 4-10 showed the time necessary for the adsorption of LPS in static contact. However, maximizing LPS capture in the flow of the mobile phase by this method becomes important for effective concentrating LPS diluted in large volume. A 10 ml sample containing 1μg LPS was passed through the PEI-cloth in the Swinnex™ cartridge (Fig. 4-11) in 30 s to 20 min once, and the captured LPS was measured by CEIA as described in the mobile phase method. From Fig. 4-12, it was found that 45 s to pass per 1 ml sample was required to achieve maximum adsorption.

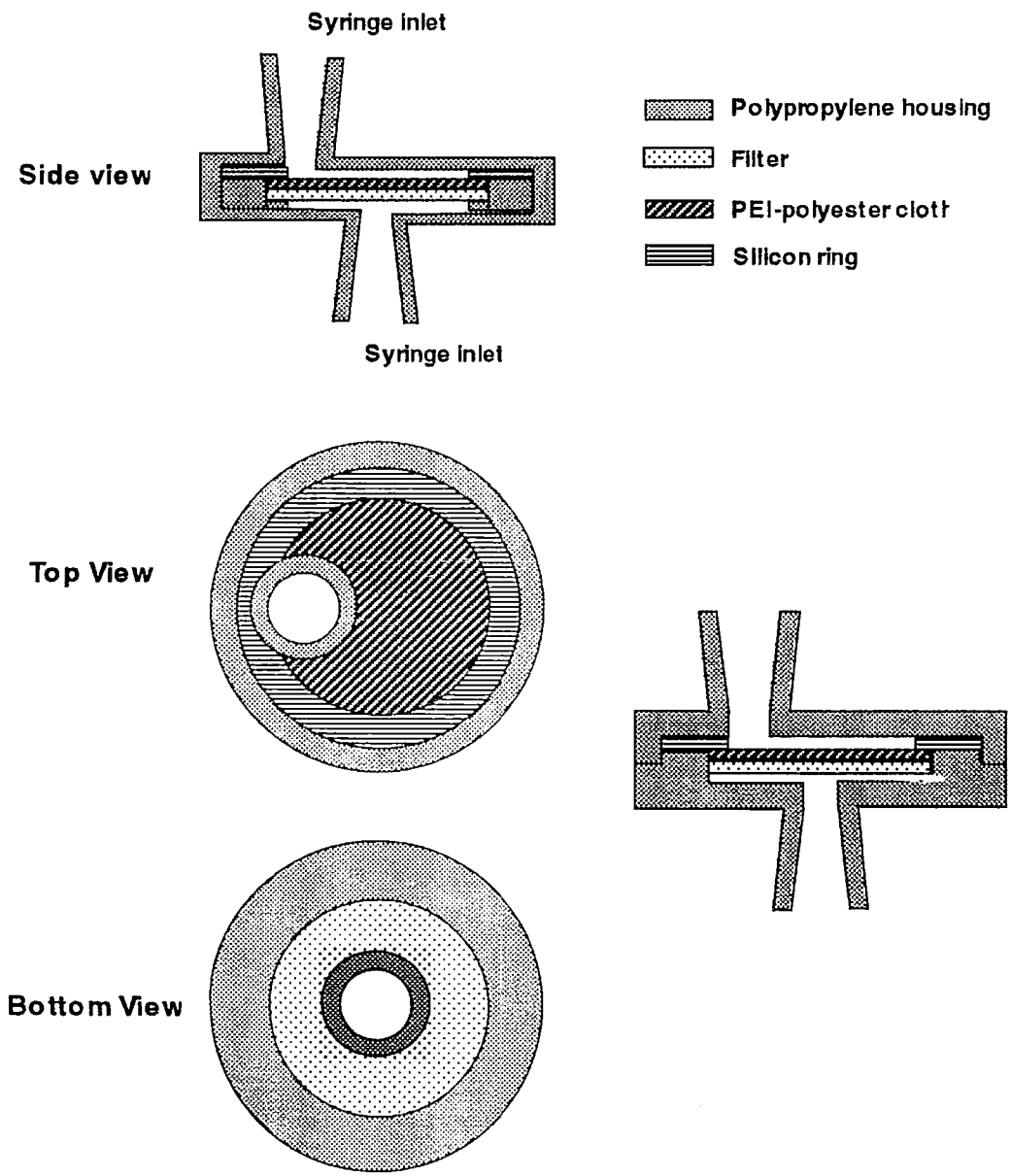


Fig. 4-11. Design of disposable poly(ethyleneimine)-cloth cartridge for PEI-cloth enzyme immunoassay

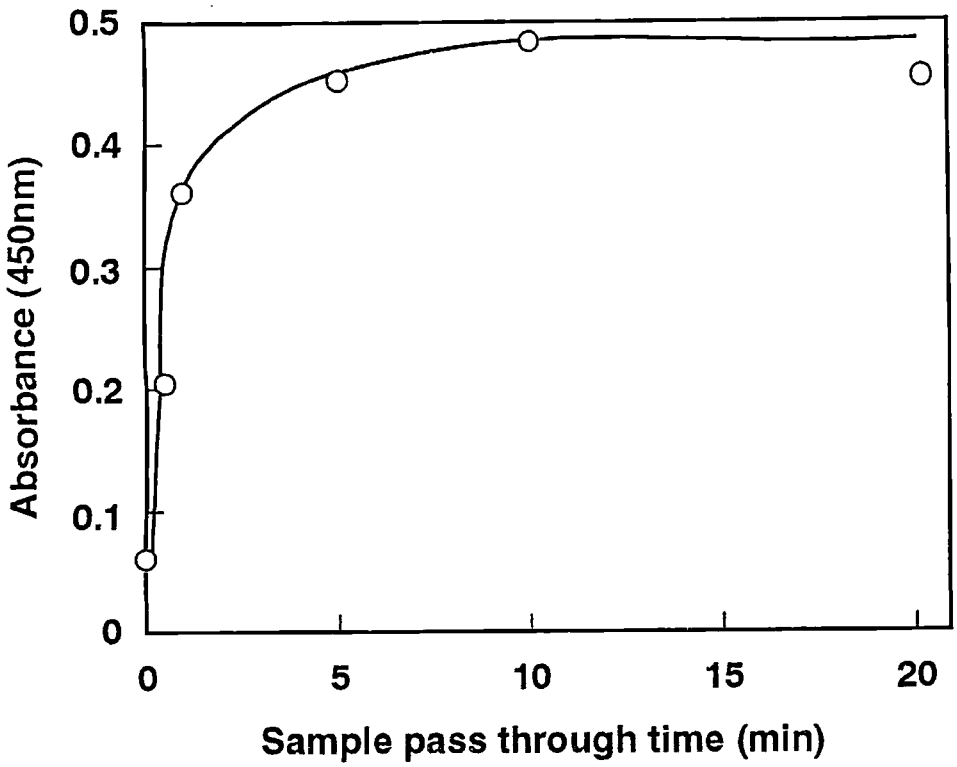


Fig. 4-12. Effect of sample passing time through cloth for capturing LPS in mobile phase.
A 6 mm diameter PEI-cloth disk set in a Swinnex 13 cartridge was passed with a 10 ml sample containing 1 μg *Salmonella* LPS in 30 s to 20 min once and then followed by CEIA. The incubation time with soluble TMB reagent was 2 min. In the figure, time (min) expresses the passing time required for 10 ml sample through a PEI-cloth cartridge.

4.9.5.3. **Detection limit by mobile phase adsorption**

This mobile phase method allows the capture of most of the LPS in the sample through concentration. This contributes to an enhanced sensitivity relative to the usual assay method, which can only utilize a small portion of the sample for assay and sometime ends up undetected. To find the effect of volumes in the adsorption with equal amounts of LPS samples of LPS from 1pg to 1 μg contained in 1 ml or 10 ml were passed through PEI-cloth, and dot blot assays were done. As seen in Fig. 4-13, 1 pg (16 cells) can be detected in a 1ml sample but 10 pg (160 cells) for a 10 ml sample. Thus the detection limit was one order lower when the volume was one order higher. The detection limit of the PEI-CEIA dot blot assay was 10 ng/ml or some cases 1 ng/ml was possible, however the present method extended the limit by about ten thousand times. The assays with soluble TMB, as shown in Fig. 4-14, confirm that the larger volume of diluted sample adsorbs less on the cloth.

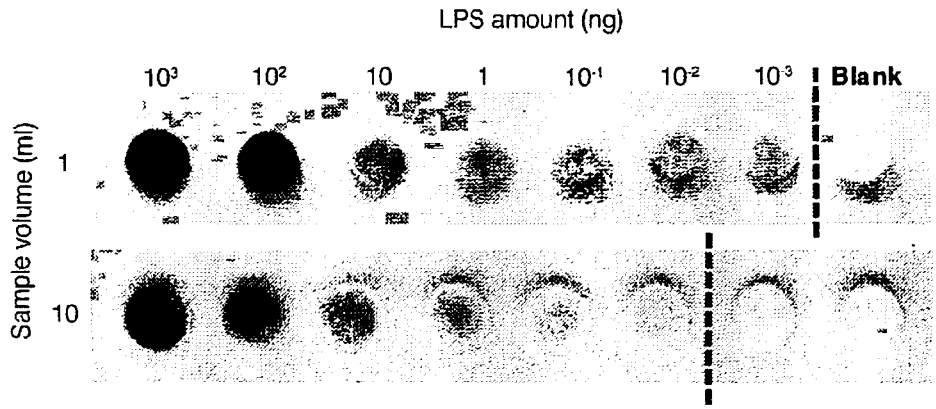


Fig. 4-13. Dot blot assay of captured dilute LPS samples in large volume samples. A PEI-cloth set in a Swinnex 13 cartridge was passed in the rate of 45s/ml with 1 or 10 ml samples containing 1 pg to 1 mg Salmonella LPS and then CEIA was followed. Salmonella LPS in 1 ml sample was detectable up to 1 pg through concentration and 10 ml sample was 10 pg.

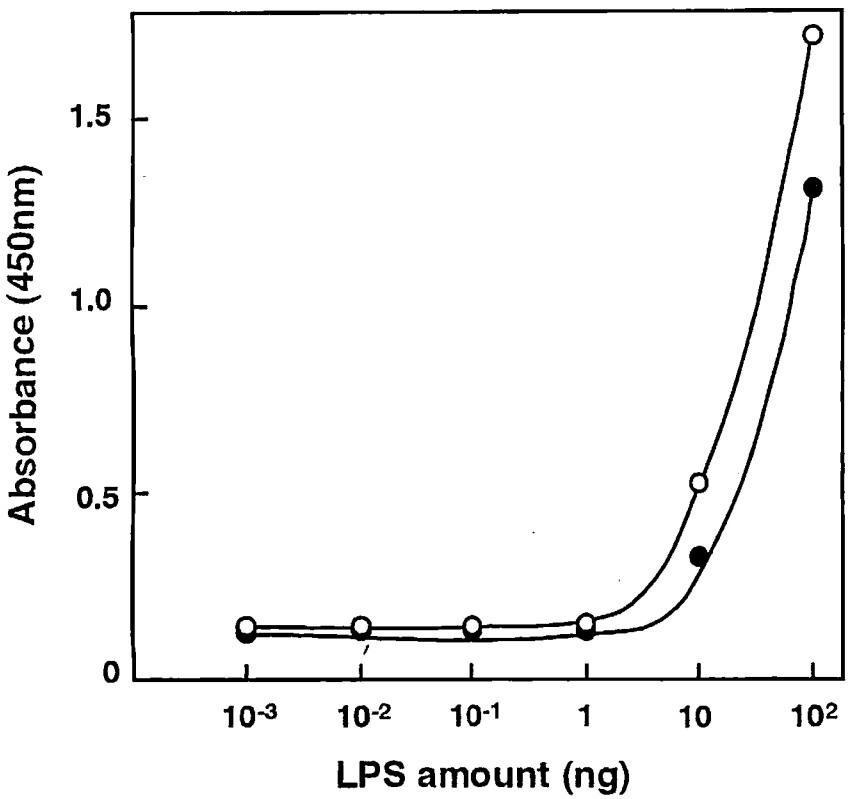


Fig. 4-14. Captured dilute LPS samples in large volumes. Under the same conditions of Fig 3, experiments, using 1 (○) or 10 (●) ml samples containing 1 pg to 100 ng *Salmonella* LPS, CEIA were carried out. The incubation with soluble TMB reagent was 10 min. The both blank absorbencies were 0.09 at 450 nm.

4.9.5.4. **Detectability of LPS in chicken soup**

As a demonstration to be able to detect LPS in a simulated contaminated food, 1 μg of *Salmonella* LPS was spiked in chicken soup supernatant (Lipton chicken soup, solid 84 mg/ml of protein, fat and carbohydrate) in 1 or 10 ml which was in a semi-clear state. As seen in Table 4-3, the soup contents resulted in LPS adsorptions of about 84% in 1 ml and 37% in the 10 ml samples, while for comparison, in the buffer about 97% and 84% were observed, respectively. The drastic decline of the adsorption in the 10 ml soup sample could have been due to the accumulation of soup material on the cloth, preventing the LPS from contacting with PEI-cloth. However it was still possible to detect LPS through concentration.

	Absorbance at 450 nm		
	Blank (no LPS) 1 ml	**LPS 1 μg in 1ml**	**LPS 1 μg in 10 ml**
In PBS containing 0.55% cholate	0.04	0.92	0.79
In chicken soup containing 0.55% cholate	0.07	0.79	0.35

Table 4-3. Salmonella LPS detection spiked in chicken soup.
A PEI-cloth set in a Swinnex 13™ was passed with 1 or 10 ml chicken soup spiked with 1 μg *Salmonella* LPS and then treated with CSA-1 HRP conjugate. The last EIA incubation in 1 ml soluble TMB reagent was 2 min. As a control for 100% adsorption, 1 μg of LPS spotted on the cloth and its CEIA done under same conditions gave 0.94 at 450 nm.

4.10. **Summary for Chapter 4**

PEI-cloth has an advantage of direct sample application to the dry-state cloth and was able to capture LPS much faster than p-cloth, e.g., PEI cloth

within 5 minutes, while p-cloth took at least 15 min., which may reduce assay time. It was found that PEI 800 bonded-cloth with incubation for 2~5 minutes (low incorporation of PEI) at 110°C gave best sensitivity by dot blot method, and able to detect a sample (5 μl) of *Salmonella* LPS 10 ng/ml (= ~1.6×10^5 cells/ml) that appeared as a weak but definite blue spot, while coated PEI-coated cloth prepared from more than 2 hr incubations at 40°C gave equivalent sensitivity with p-cloth detected 100 ng/ml with clear blue spot but no spot was observed below 10 ng/ml. It was also possible to detect *Salmonella* LPS in the presence of non-*Salmonella* LPS in 100 times excess. We have demonstrated that PEI-cloth is suitable as a specific adsorbent for *Salmonella* LPS and permits the qualitative and quantitative assays in the identifications of *Salmonella* and other Gram-negative bacteria by PEI-CEIA. By rapid LPS detection method, PEI-cloth in a cartridge concentrated dilute *Salmonella* LPS in large volumes efficiently passed by syringes or a peristaltic pump for enzyme immunoassay. The enhanced sensitivity by PEI-cloth itself and its efficient concentration made possible to detect 1 pg LPS which is equivalent to about 16 bacteria and would contribute to a shortening of the pre-incubation time of cultures and consequently the total detection time. This method can equally be applied to the detection of other Gram negative bacteria.

4.11. **References for Chapter 4**

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Chapter 5. **Design and preparation of LPS and DNA adsorbing PEI beads**

5.1. **Background for designing LPS and DNA adsorbing PEI beads**

Cell products for medical use often contaminated with recombinant DNA, although the effect on human is not yet certain. It is desired to remove DNA selectively from vaccine products without losing effectual ingredients. WHO determines the maximum DNA content in one dose of vaccine must be 10 ng (1). DNA has phosphate groups similar to LPS and should be adsorbed onto polyamine-spherical beads which are prepared for LPS removal. In fact. Ida et al (2) reported that chitosan beads had high adsorption of LPS, and it should adsorb DNA with high capacity through inter actions between phosphate groups of DNA and amine groups of beads. Based on this, PEI cross-linked spherical beads which were designed and prepared for selective removal of LPS were applied for selective adsorption of DNA as well (3).

5.2. **Materials**

A 30 wt-% PEI aqueous solution (degree of polymerization: 1600. M_w: 70000) was purchased from Wako Pure Chemical (Osaka, Japan). CMO was purchased from Nacalai Tesque (Kyoto, Japan). The purified nucleic acids (DNA from salmon spermary (M_w: 3 x 10^5) and RNA from yeast (M_w: 2.5 x 10^5)) were purchased from Wako Pure Chemical Ind. Ltd., Osaka. Egg albumin (M_w: 4.5 x 10^4, pI: 4.6), bovine serum albumin (BSA) (M_w: 6.9 x 10^4, pI: 4.9), myoglobin (from horse heart, M_w: 1.8 x 10^4, pI: 6.8), γ-globulin (from human serum, M_W: 1.6 x 10^5, pI: 7.4), cytochrome C (from horse heart, M_w: 1.3 x 10^4, pI: 10.6), and lysozyme (from, M_w: 1.5 x 10^4, pI: 11) were purchased from Sigma Chemical Co. Ltd. The fluorescent dye 4',6-diamidino-2-phenylindole dihydrochloride hydrate for fluorometric analysis (4) was purchased from Nacalai Tesque. The chitosan particles (Kurimuver II) (5. 6) were purchased from Kurita Water Industries Ltd., Tokyo and used as a standard adsorbent.

5.3. **Preparation of Adsorbents, PEI beads**

A new PEI beads preparation was designed to remove endotoxin (LPS) from medicine and blood products. LPS causes fever, endotoxin shock and in worst case death when it is administered especially intravenously. PEI contains amine group in the structure which enable to prepare beads with bridging agent such as CMO in one step without adding the functional group for it. The reaction between ammonia and epoxides is a general reaction and very useful method to prepare β-hydroxyamines as seen in Fig. 5-1, basically gives the primary amines but some secondary and tertiary amines. Thus primary and secondary amines respectively give secondary and tertiary amines (7).

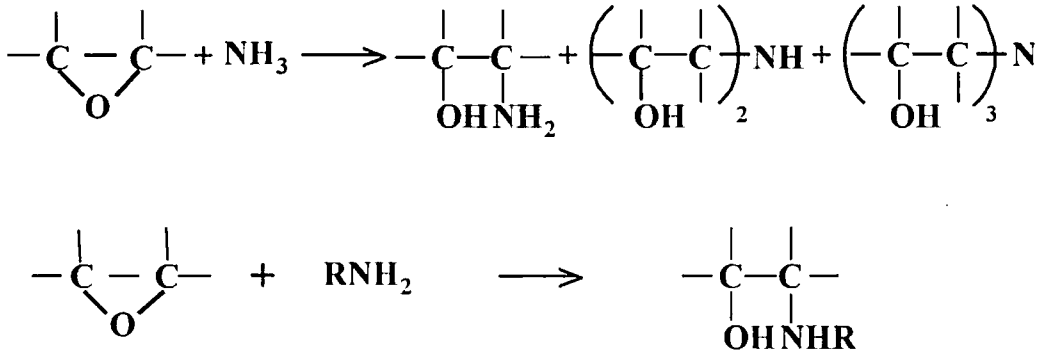

Fig. 5-1. Amine and epoxide reactions

The advantages to use PEI to prepare beads by this reaction are that constituting unit of PEI is rather small molecular weight of 43 which offers more available ionic interaction sites and commercially available in many different molecular weights, which makes possible to adjust easily in hydrophobic and hydrophilic nature of the beads.

PEI/CMO spherical particles were prepared by suspension cross-linking (Fig. 5-2) as follows: A 30 wt-% PEI aqueous solution and cross-linking agent of CMO were mixed at 0 °C. The mixture was suspended in a paraffin liquid. The suspension was stirred at 0 °C for 30 min and then at 80 °C for 24 h. The particles obtained were washed successively with acetone, chloroform,

methanol, ethanol and distilled water. All particles obtained with diameters from 44 to 105 μm were used as adsorbents.

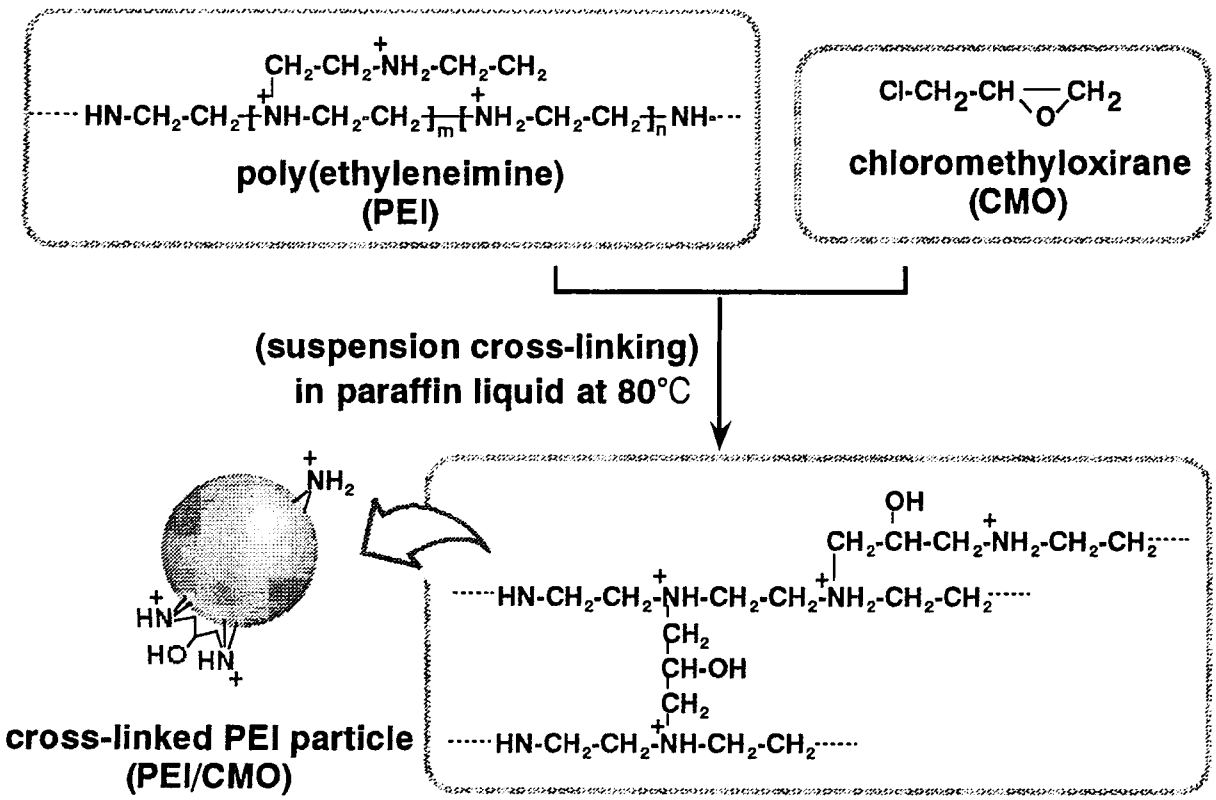

Fig. 5-2. Preparation of cross-linked poly(ethyleneimine) (PEI/CMO) spherical particles and their structure and charge distribution.

5.4. **Characterization of PEI spherical beads**

The pore size of the matrix in the adsorbent was estimated as the molecular mass exclusion (M_{lim}) from the calibration curves by aqueous size exclusion chromatography (8). The M_{lim} values were reduced as the molecular mass of polysaccharide. Homogeneous series of the polysaccharides (pullulan and maltose) were used as the permeable substances. The amino-group content

of the adsorbent was quantified by pH titration and by elemental analyses.

In PEI and CMO reaction, mixtures 90/10 to 70/30 had gave good matched products with theoretically estimated values in C/N ratio but 50/50 product was very close to 70/30 as seen in Table 5-1. This indicates that 70/30 mol% ratio is the maximum effective one to prepare CMO bridged beads.

PEI/CMO mixed		90/10	80/20	70/30	50/50
C (%)		44.4	47.6	45.4	45.3
H (%)		10.4	9.2	9.1	8.7
N (%)		22.9	20.4	15.9	15.2
C/N	Thoretical	2.0	2.4	2.8	4.3
	Found	1.9	2.3	2.9	3.0
PEI content (%) found		93	81	68	67

Table. 5-1. Element Analysis of PEI Spherical Beads

PEI Sherical Beads	AEC (meq/g)	Sd (wet-ml/dry-g)	pK_a	M_{lim}
PEI/CMO=93/7	7.8	15.4	8.0	9000
PEI/CMO=81/19	7.1	5.8	8.0	1400
PEI/CMO=68/32	2.9	3.0	7.8	660
PEI/CMO=67/33	2.3	2.4	7.9	300

AEC: Anion Exchange Capacity, Sd: Swelling degree

Table. 5-2. Characterization of PEI Spherical Beads

In PEI/CMO ratio effect, as CMO increases, anion exchange capacity (AEC) decreased (Table 5-2). This was due to the aliphatic group content of a bead surface increased and consequently the surface amine content decreased in relative. The swelling degree (S_d) decreased as CMO increased with more cross linking occurred and the hydrophilic amine group decreased in relative and thus the structure become more rigid. The pKa remained very close each other between 7.8 to 8.0 possibly because no other functional groups except primary to tertiary amines influences on total surface charges. The porosity was measured as M_{lim} (molecular limit) using sugar and polysaccharides by size exclusion chromatography (SEC). As CMO portion increases, M_{lim} decreased because of more cross linking and forming rigid structure.

5.5. **PEI beads as LPS adsorbent**

5.5.1. **LPS and Proteins adsorption onto PEI beads**

5.5.1.1. **LPS measuring method adsorbed on PEI bead**

PEI beads were washed with methanol, 0.1M NaOH, 2M NaCl and water successively and finally equilibrated with PBS. All reagents were prepared with LPS free water. An amount of 0.2 g PEI bead was weighed in a 50ml Erlenmeyer flask and shaken slowly with 2 ml LPS solution in PBS for 2 hrs. The liquid portion was taken by a syringe and filtered through 0.45 μm membrane filter. The unbound LPS was determined by Limulus Test.

5.5.1.2. **Protein measuring method adsorbed onto PEI bead**

Adsorption experiments of protein onto PEI beads was done same way as LPS adsorption but last filtering was done through 0.8 μm membrane filter. The unbound protein was measured by UV absorbency at 278 nm for BSA (Mw. 69,000, pI 4.9) and γ-globulin (Mw, 160,000, pI 7.4), and 409 nm for cytochrome C (Mw 13,000, pI 10.6).

5.5.1.3. **LPS and protein adsorption behavior onto various PEI beads**

LPS and proteins were individually studied for their adsorption behaviors. As seen in Fig. 5-3, LPS and BSA adsorption increased as PEI content in the bead increased while γ-globulin, a neutral protein, which adsorption is more based on hydrophobicity, declined. 80 mol% beads had good LPS adsorption especially when LPS content is low (!00 ng/ml) at physiological conditions, but 90 mol% bead was not suitable because it was not solid enough. On the other hand, BSA at 100 μg/ml had rather high adsorption of 38 mol%, which adsorption is more based on ionic binding could go up at lower concentration (eg. 100 ng/ml), while γ-globulin at lower concentration (100 ng/ml) may be even less in adsorption %. These studies were done under low ionic strength of μ=0.05 lower than physiological condition. Under higher ionic strength, proteins adsorption could be less.

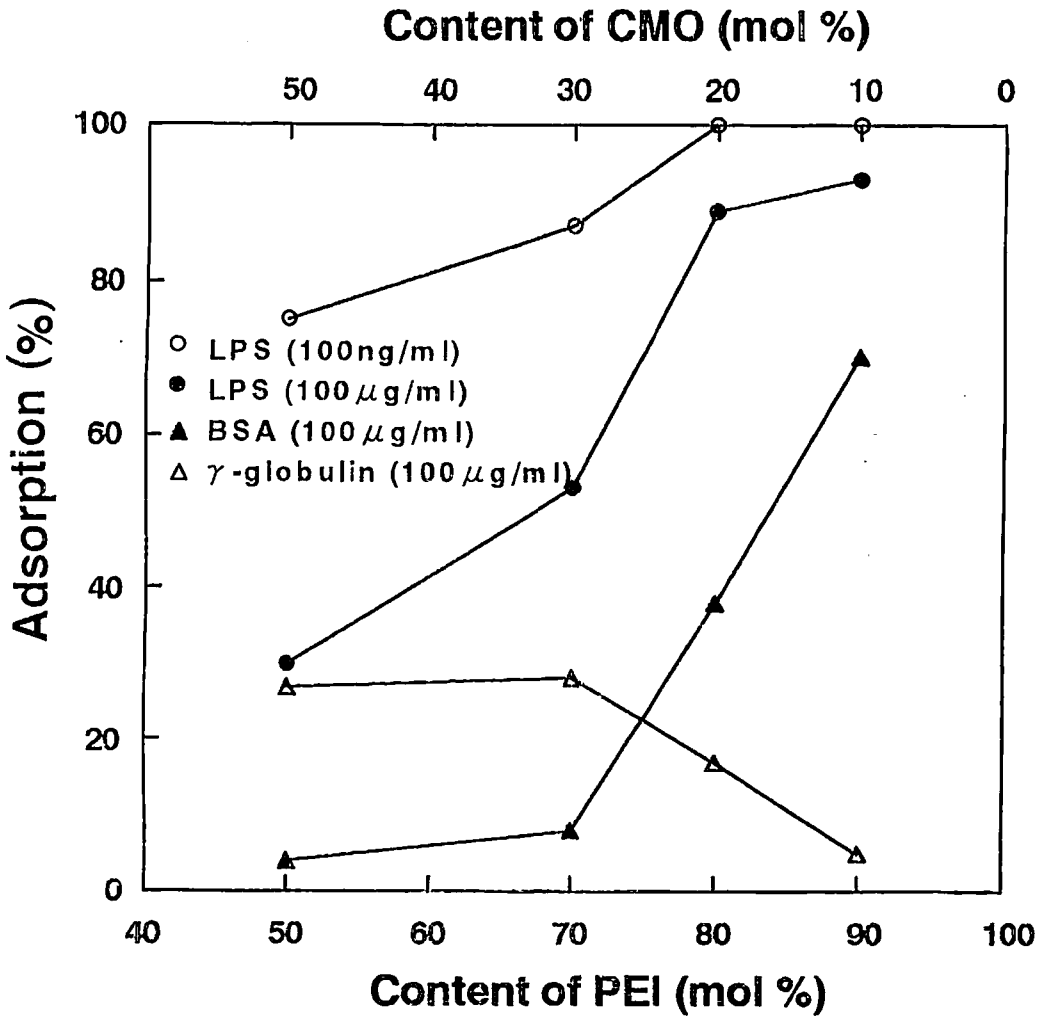

Fig. 5-3. Effect of PEI content in PEI beads on adsorption of LPS and proteins at pH 7.0 and μ= 0.05. Adsorbent 0.2 wet-g per 2 ml sample solution was used.

5.5.1.4. **The ionic strength effect on adsorptions of LPS and proteins**

As PEI bead of PEI/CMO=90/10 was too soft to use as a bead, PEI/CMO=80/20 bead was used for the study. The effect of ionic strength on the adsorption of above compounds at 100μg/ml, pH 7. As ionic strength μ

=0.05 ~ 0.8 increased, LPS and BSA went down in adsorption drastically but LPS adsorption went up at the end ionic strength (Fig. 5-4). This phenomenon could be due to more hydrophobic adsorption start to take over. γ-globulin was constantly in low adsorption. This could be globulin is adsorbed as weak hydrophobic protein and no ionic adsorption is involved.

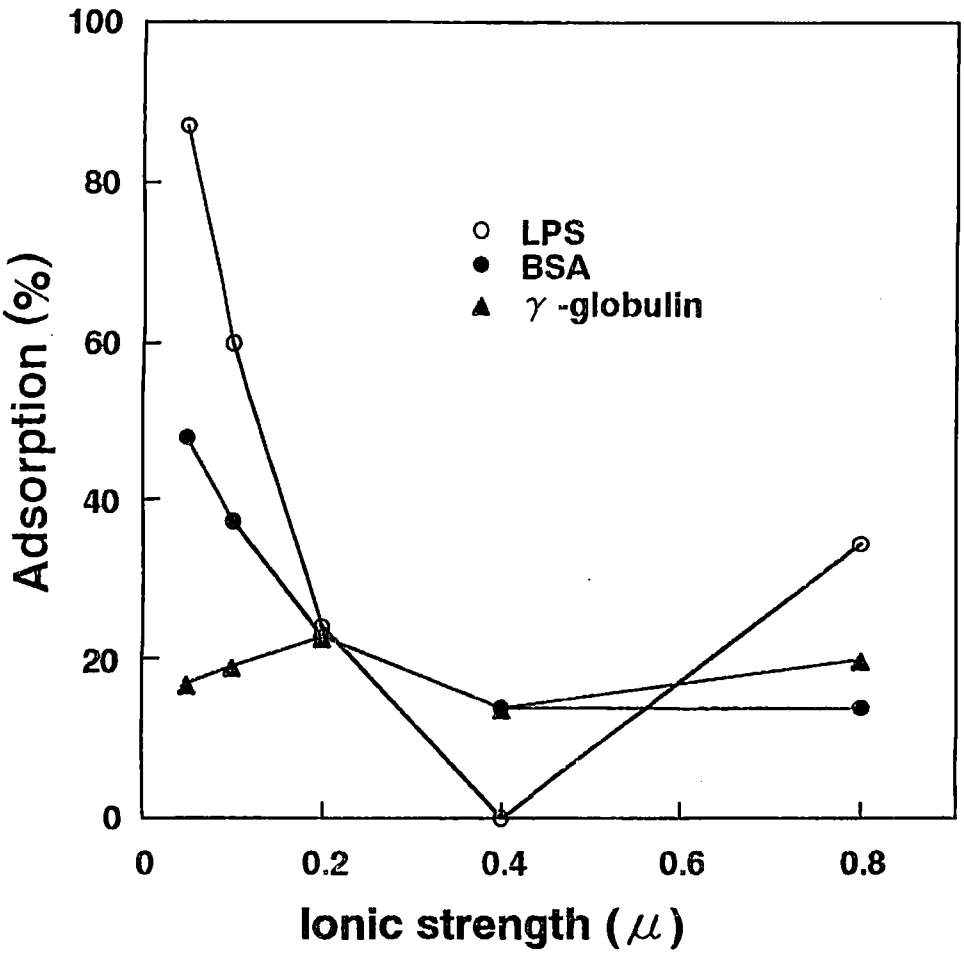

Fig. 5-4. Effect of ionic strength (μ) on adsorption of LPS, BSA and γ-globulin using PEI/CMO (80/20) beads at pH 7

5.5.1.5. **LPS adsorption in the presence of proteins.**

As proteins, BSA (Mw, 69,000, pI 4.9), γ-globulin (Mw, 160,000, pI 7.4), and cytochrome C, (Mw 13,000, pI 10.6) were 1mg/ml each and LPS was 100ng/ml in phosphate buffer pH 7.2, μ=0.17 using PEI/CMO=80/20 bead. The LPS adsorbing experiment was done by batchwise method with 0.2 g of wet adsorbent and 2 ml of a protein solution. The PEI beads showed very high adsorption % from a protein contained solution (above 99%) while in case of the acidic protein BSA adsorption was kept low adsorption.

This result shows that this PEI beads can remove LPS in low concentration (100ng/ml) contained in high concentration of protein 1 mg/ml quite efficiently as seen in the Table 5-3 and Fig. 5-3.

Sample solution	LPS removal		Recovery of protein (%)
	Residual amount (ng/ml)	Removal rate (%)	
LPS	<0.01	>99	—
BSA & LPS	1.0	99	99
γ-globulin & LPS	0.5	99	99
cytochrome C & LPS	0.4	99	99

Table 5-3. Selective removal of LPS from protein solution by PEI (PEI/CMO=80/20)beads. The removal of LPS was determined by a batchwise method using 0.2 g wet beads and 2 ml solution mixture of a protein (1mg/ml) and LPS (100 ng/ml) at pH 7.2, μ=0.17.

5.5.2. **Summery for LPS adsorption onto PEI beads**

The Anion Exchange Capacity increased from 2.3 to 7.8 meq/g as increased PEI incorporation from 50 to 90 mol% in bead. Same tendency occurred in the swelling rate and Mlim. PEI/CMO (90/10) and (80/20) beads at pH 7.0, μ= 0.05 had high LPS adsorption of above 85%. With PEI/CMO (80/20) beads under physiological condition study at pH 7.2, μ= 0.17, LPS removal rate was

above 99% and unbound protein recovery rate was more than 95%. The PEI beads had good selective adsorption on LPS but not on proteins.

5.6. **PEI Spherical beads as DNA adsorbent**

5.6.1. **DNA and Protein Assay**

The DNA concentration in the sample solution was determined by fluorometric analysis (9) with a Spectrofluorophotometer FP-6500 (JASCO) using the fluorescent dye 4',6-diamidino-2-phenylindole dihydrochloride hydrate. The protein concentration was measured at 280 nm with a Spectrophotometer UVIDEC-660 (JASCO).

5.6.2. **Adsorption method of DNA and other cellular products onto PEI bead**

Nucleic acids (DNA or RNA) were dissolved in the following buffers: 0.02 M sodium acetate (pH 4, 5); 0.02 M phosphate (pH 6, 7, 8); 0.02 M Tris (pH 9). The ionic strength of the buffer was adjusted by changing the content of sodium chloride. The adsorption of nucleic acids was measured by a batchwise method as follows: the adsorbent was washed and equilibrated with various buffers with different pH and ionic strengths. A 0.1- to 0.4-g portion of wet adsorbent was suspended in 2-4 ml of DNA or RNA solution. The suspension was shaken for 2 h at 25 °C and filtered through a Millipore filter (0.8 μm) to remove the adsorbent. The DNA content of the filtrate was determined. The apparent dissociation constant ($K_{d,app}$) between the DNA and the adsorbent was estimated by adsorption isotherm (10,11). The adsorption of cell products other than DNA was investigated by a method similar to DNA-binding assay.

5.6.3. **Dissociation constant between DNA and the adsorbent**

In order to achieve the selective adsorption of DNA, it is important to decrease the interaction between the adsorbent and other cellular products, such as protein. It was found that the protein-adsorbing activity of the aminated

poly(γ-methyl L-glutamate (PMLG) adsorbent increased drastically with increasing pore size in the adsorbent's matrix to sizes over the molecular weight of the protein (12). Furthermore, it was clear that the adsorbent showed good DNA-adsorbing activity, even at small pore sizes (below 10^4 as M_{lim} of polysaccharides) (10). These results suggest that adsorbents with M_{lim} below 10^4 would adsorb DNA selectively from the protein solution. Therefore, various cross-linked PEI (PEI/CMO) particles with pore sizes of M_{lim} $5x10^2$ to $9x10^3$ were prepared as adsorbents (Table 5-4). The amino-group content of the adsorbent was easily adjusted by changing the PEI ratio and the CMO ratio in cross-linking. When the unit-molar ratio of CMO (in cross-linking) increased from 10 to 50 %, the amino-group content of the particles, their pore size, and their degree of swelling (in water) also decreased from 7.8 to 2.1 meq g^{-1}, from $9x10^3$ to $5x10^2$, and from 15.4 to 2.4 wet-mL/dry-g, respectively. Thus, an increase in the content of the cross-linking agent leads to an increase in the hydrophobicity of the adsorbent, resulting in a decrease in its degree of swelling in water.

Table 5-4 . Characteristics of cross-linked PEI adsorbents

Adsorbent	Molar ratio mol%		Amino-group content[b]	Pore size of matrix	S_d[d] wet-ml/dry-g	Adsorbing capacity[e] of DNA	$K_{d,app}$[e] of DNA
	PEI[a]	CMO	meq g^{-1}	M_{fim}[c]		mg mL^{-1} adsorbent	M
PEI/CMO=90/10	90	10	7.8	9×10^3	15.4	5.5	9.5×10^{-10}
PEI/CMO=80/20	80	20	7.1	2×10^3	5.8	5.4	1.8×10^{-9}
PEI/CMO=70/30	70	30	2.9	1×10^3	3.1	3.2	3.5×10^{-9}
PEI/CMO=60/40	60	40	2.5	1×10^3	2.7	2.9	8.5×10^{-8}
PEI/CMO=50/50	50	50	2.1	5×10^2	2.4	2.7	9.3×10^{-8}
chitosan particles[f]	_	_	3.6	1×10^6	16.5	17.0	9.3×10^{-9}

[a] Unit-mol % of PEI in the adsorbent (1 unit: -(CH_2-CH_2-NH)-; degree of polymerization: 1600).

[b] Content of amino groups in the adsorbent.

[c] Value deduced as a molecular weight of polysaccharide (10).

[d] Degree of swelling in water.

[e] The DNA-adsorption capacity per ml adsorbent and the apparent dissociation constant ($K_{d,app}$) of DNA to adsorbent were estimated by adsorption isotherm, as described previously (3,6). The adsorption isotherm of DNA was determined using a batchwise method with 0.1 ml of wet adsorbent and 4 ml of a DNA solution (DNA from salmon spermary (M_w 3 x 10^5): 1 to 1000 μg ml^{-1}, pH 7.2, μ = 0.17). The $K_{d,app}$ was expressed in mol L^{-1} (M) of molecular weight of DNA.

[f] Kurimover-II (4,5)

In the removal of DNA from a dilute DNA solution by adsorption, the DNA-removing activity depends more on the apparent dissociation constant ($K_{d,app}$) between DNA and the adsorbent than on the adsorbing capacity. The adsorbing activities of the adsorbents for DNA were examined by a batchwise method with various kinds of buffers. The purified DNA (from salmon spermary) was used as a standard DNA sample. As shown in Table 5-4, while the PEI ratio increased from 50 to 90 unit-mol%, the DNA-adsorbing capacity increased from 2.1 to 5.5 mg (as purified DNA from salmon spermary, M_w: $3x10^5$) per ml of wet adsorbent and the $K_{d,app}$ of DNA to the adsorbent decreased remarkably from $9.3x10^{-8}$ to $9.5x10^{-10}$ M, under physiological conditions (ionic

strength, μ= 0.17, pH 7.2).

Although chitosan particles showed the largest DNA-adsorbing capacity (17.0 mg mL^{-1}) because of the entry of the DNA into the pores (M_{lim} $1x10^6$) of the particles. PEI/CMO adsorbents with PEI ratios of 90 unit-mol% (PEI/CMO= 90/10) had the smallest $K_{d,app}$ values ($9.5x10^{-10}$ M). Even in PEI/CMO= 80/20 and 70/30 adsorbents, the $K_{d,app}$ value of each adsorbent was smaller than that of the chitosan particles.

The smaller the $K_{d,app}$ value, the stronger the DNA-removing activity of the adsorbent (11). These results indicate that the DNA-removing activity of PEI/CMO adsorbents (PEI ratio of 70 to 90 unit-mol%) are higher than those of chitosan particles when the removal of DNA from a dilute DNA solution is attempted at μ= 0.17 and pH 7.2.

5.6.4. **Effects of Various Factors on Adsorption of DNA and Protein**

For the selective adsorption of DNA, it is necessary to check the interaction between the adsorbent and cellular products. Fig. 5-5 shows the effect of PEI content in the adsorbent on adsorption of cellular products (DNA from salmon spermary, BSA, and γ-globulin) at pH 7.0 and an ionic strength of μ= 0.05. The DNA-adsorbing activity increased from 63 to 99 % with an increase in PEI ratio from 50 to 80 unit-mol %.

The PEI/CMO=80/20 and 90/10 adsorbents with high PEI ratio (80 and 90 unit-mol %, respectively) showed excellent adsorbing activity: They were able to decrease the concentration of DNA from 500 μg/ml to less than 10 ng/ml. The adsorption of BSA was also increased from 2 to 34 %, with an increase in PEI ratio from 60 to 90 unit-mol%. By contrast, the adsorption of γ-globulin increased from 2 % to 35 with a decrease in the PEI ratio from 80 to 50 unit-mol % (with an increase in the ratio of CMO from 20 to 50 mol%). Little, basic protein such as lysozyme, was adsorbed (<2 %) by any of the PEI/CMO adsorbents.

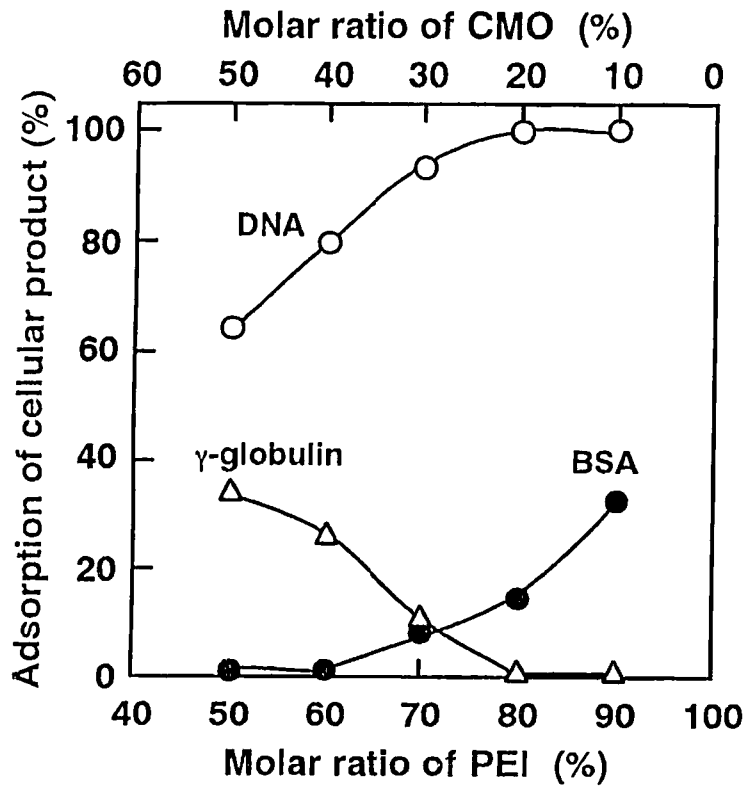

Fig. 5-5. Effect of molar ratio of PEI in adsorbents on the adsorption of cellular products. The adsorption of a cellular product was determined by a batchwise method with 0.2 ml of wet adsorbent and 2 ml of a sample (500 μg/ml) solution (pH 7.0, μ= 0.05).

The effects of ionic strength and pH on DNA adsorption by PEI/CMO=80/20 and 90/10 adsorbents were compared with those of the chitosan particles (Fig. 5-6a and b). As shown in Fig. 5-6a, the higher the ionic strength of the buffer, the lower the DNA-adsorbing activity of each adsorbent. At high ionic strengths of μ= 0.6 to 1.0, chitosan particles always showed greater DNA-adsorbing activity.

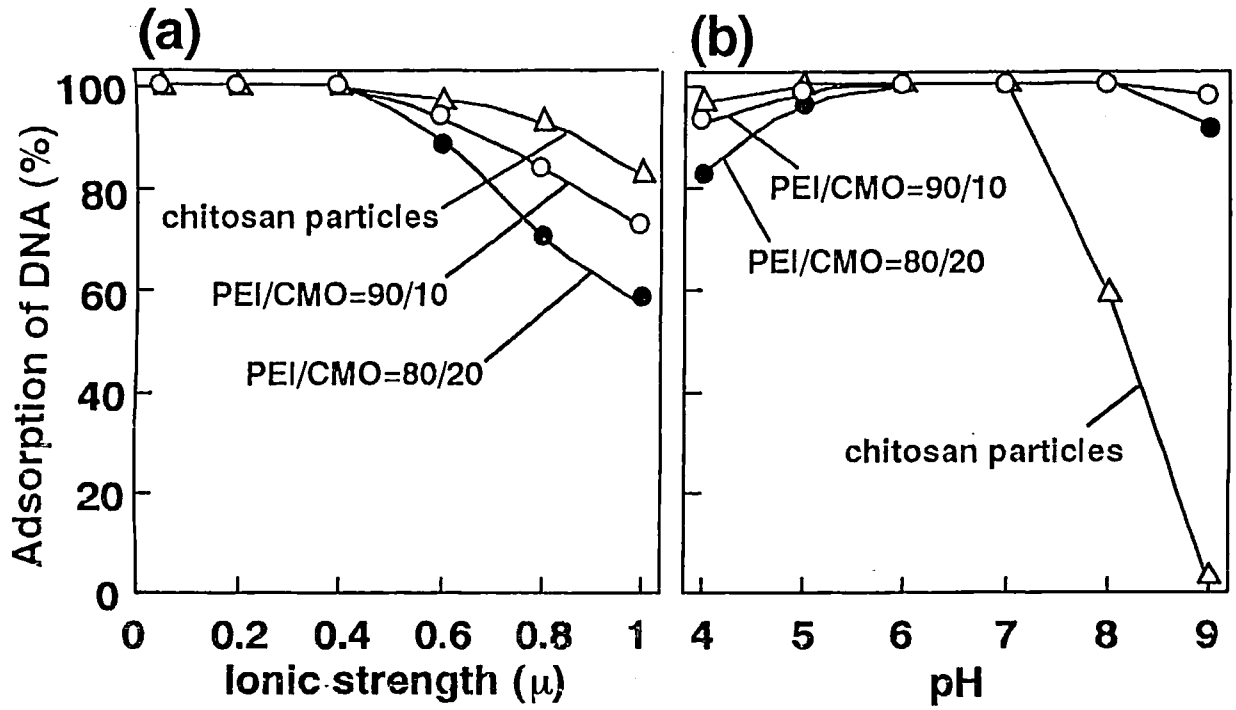

Fig. 5-6. Effects of the buffer's ionic strength (a) and its pH (b) on the adsorption of DNA by PEI/CMO=80/20, 90/10, and chitosan adsorbents. The adsorption of DNA was determined by a batchwise method with 0.2 ml of wet adsorbent and 2 ml of a DNA solution (purified DNA from salmon spermary: 500μg/ml).

For a wide pH range from 4 to 9, as shown in Fig. 5-7b, each PEI/CMO adsorbent showed a high DNA-adsorbing activity (82 - 99%). Higher DNA-adsorbing activity was observed in the PEI/CMO=90/10 with the larger PEI content. On the other hand, chitosan particles showed high adsorption for DNA only in the range of pH 4 to 7. This result shows that DNA is bound more strongly by PEI/CMO than by chitosan particles at high pH (8 and 9). This difference is attributable to the fact that the pK_a of the PEI (pK_a: 8.7) is higher than that of chitosan (pK: 6.2). This is because the useful pH range for DNA adsorption increased with increasing pK_a of the adsorbent. As a result, it was found that PEI/CMO=80/20 and 90/10 showed high DNA-removing activity at

ionic strengths of μ= 0.05 to 0.4 and over a wide range of pH 4 to 9.

The effect of the ionic strength of the buffer on the adsorption of BSA and γ-globulin was examined with various PEI/CMO adsorbents at μ= 0.05 to 0.8 and pH 7.0 (Fig. 5-7a and b). The BSA-adsorbing activity of the adsorbent increased with decreasing ionic strength, as shown in Fig. 5-7a. At ionic strengths of μ= 0.05 to 0.2, PEI/CMO=90/10, with the largest PEI content, always showed the highest BSA-adsorbing activity. When the ionic strength was increased to μ= 0.4 or higher, the BSA adsorption was < 2% in all of the adsorbents. By contrast, PEI/CMO=50/50, with the largest CMO content, always showed the highest γ-globulin-adsorbing activity at all ionic strengths (Fig. 5-7b): 38 to 43% of γ-globulin adsorption was adsorbed. The adsorption by both PEI/CMO=80/20 and 90/10 was found to be less than 2% under similar conditions.

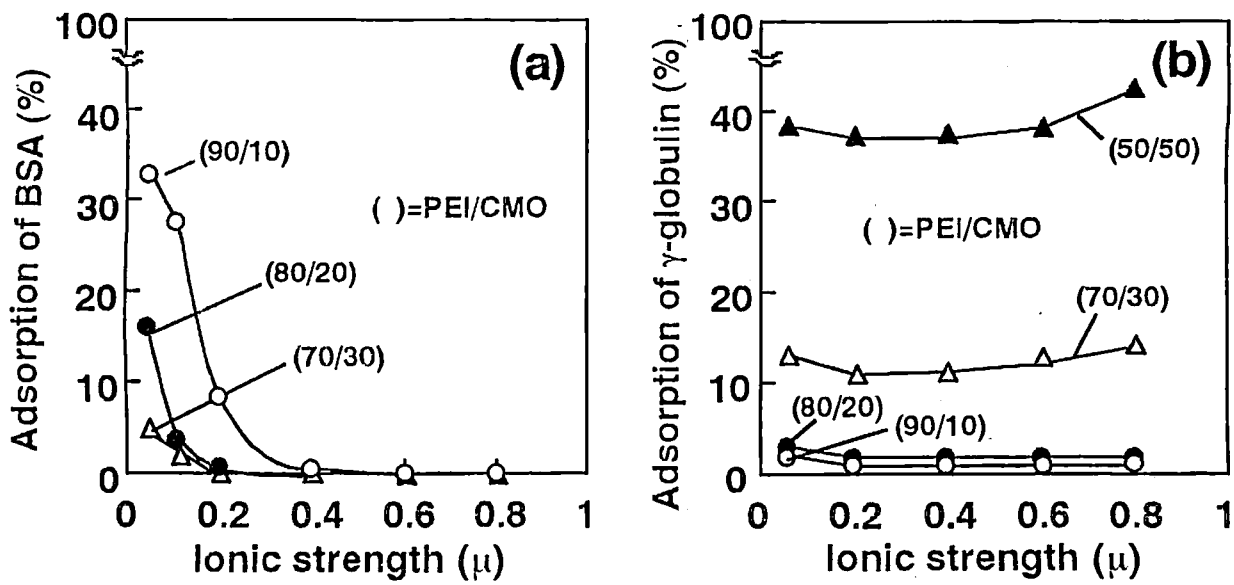

Fig. 5-7. Effect of a buffer's ionic strength on the adsorption of (a) BSA and (b) γ-globulin by various PEI/CMO adsorbents. The adsorption of the protein was determined using a batchwise method with 0.2 g of the wet adsorbent and 2 ml of a protein solution (500μg/ml, pH 7.0, and ionic strength of μ= 0.05-0.8).

Table 5-5 shows the adsorption activities of the cell products by PEI/CMO=80/20 and 90/10 adsorbents, under the physiological conditions of pH 7.2 and μ= 0.17. The binding force of each adsorbent to the DNA, RNA, and LPS was stronger than to the protein (pI: 4.6 – 11.0). As for PEI/CMO=80/20, each protein was hardly adsorbed (<3%). On the other hand, PEI/CMO=90/10 adsorbed acidic proteins, such as egg albumin (15%) and BSA (17%).

Table 5-5. Adsorption of cellular products by PEI/CMO adsorbents

Sample		Adsorption[a] (%) (pH 7.2, ionic strength μ = 0.17)	
	pI	PEI/CMO=80/20	PEI/CMO=90/10
Egg albumin	4.6	3	15
BSA	4.9	2	17
Myoglobin	6.8	<1	<1
γ-Globulin	7.4	2	2
Cytochrome C	10.5	<1	<1
Lysozyme	11.0	<1	<1
LPS (*E. coli* UKT-B)		84	91
LPS (*E. coli* O111:B4)		90	95
DNA (salmon spermary)		99	96
RNA (yeast)		94	85

[a] The adsorption of a cellular product was determined using a batchwise method with 0.2 ml of wet adsorbent and 2 ml of a sample solution (100 μg ml^{-1}, pH 7.2, ionic strengths of μ = 0.17).

From these results (Fig. 5-5 to 5-7 and Table 5-4 and 5-5), it is assumed that the adsorbing-activity of PEI/CMO adsorbents for cellular products was induced by the simultaneous effects of their cationic properties and hydrophobic or other properties. Nucleic acids (DNA and RNA) are polynucleosides having anionic regions (phosphate groups), pentoses, purine bases, and pyrimidines, and thus the charge of DNA is anionic at pH values greater than its pK_a (<2). The charge of BSA is also anionic at pH values greater than 4.9 (its pI). The adsorption of DNA and BSA increased with increasing PEI content of the adsorbent (Fig. 5-5). It is also dependent on the ionic strength and pH of the buffer (Fig. 5-6 and 7a, respectively). This suggests PEI/CMO adsorbents adsorb DNA and BSA mainly by ionic interaction. On the other hand, the ionic interaction of the adsorbent with γ-globulin (pI 7.4) is not induced at pH 7.0 since the charge of the protein is cationic at a pH under its pI value. γ-globulin is a weakly hydrophobic protein, and its adsorption by PEI/CMO adsorbent increased with an increase in hydrophobicity (content of cross-linking agent) of the adsorbent (Fig. 5-5 and 7b). These findings suggest the participation of hydrophobic binding.

Furthermore, as shown in Table 5-5, the PEI/CMO adsorbents bind more strongly with DNA than protein. This is because the pK_a of the phosphate residues of DNA is lower than the pI of protein (pI: 4.6-11.0), and probably because the DNA was adsorbed by the adsorbent, through its multipoint-attachment onto the PEI chain (a polycation) of the adsorbent surface. As a result, PEI/CMO=80/20 can selectively adsorb DNA under physiological conditions, without the adsorption of protein.

5.7. **Removal of DNA from Various Protein Solutions**

For the selective adsorption of DNA, it is necessary not only to select the ligand of the adsorbent and its pK_a but also to control the conditions of the buffer (pH and ionic strength). The effect of the ionic strength of the buffer on the selective adsorption of DNA from a BSA-containing solution was examined with the PEI/CMO=80/20, 90/10, and chitosan adsorbents (Fig. 5-8a, b, and c). A BSA solution (500 μg/ml), to which was added purified DNA (10 μg/ml),

was used as a sample solution at pH 7.0 and an ionic strength of μ= 0.05 to 0.8. As shown in Fig. 5-8a, PEI/CMO=80/20 selectively adsorbed DNA from a BSA solution at μ= 0.1 to 0.4, without the adsorption of BSA. PEI/CMO=90/10 was able to adsorb DNA selectively at μ= 0.4 to 0.8 (Fig. 5-8b). The chitosan particles showed adsorbing activities for both DNA (96 -99%) and BSA (9 - 23%) at a wide range of ionic strengths, μ= 0.05 to 0.8 (Fig. 5-8c).

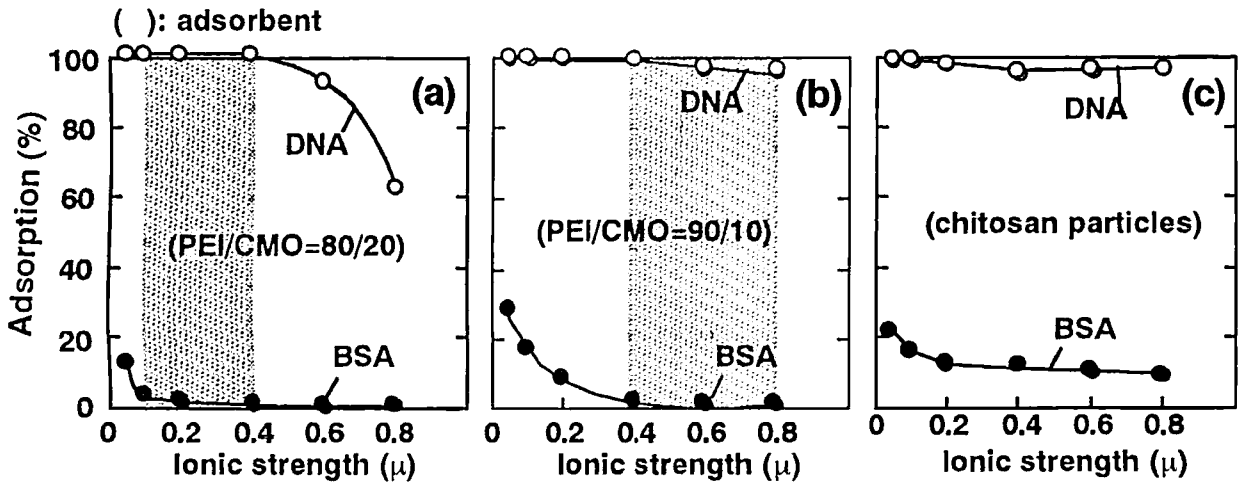

Fig. 5-8. Effect of a buffer's ionic strength on selective adsorption of DNA from a BSA solution (500μg/ml, pH 7.0, and ionic strength of μ= 0.05-0.8)) containing DNA (10μg/ml) by (a) PEI/CMO=80/20, (b) 90/10, and (c) chitosan adsorbents. The selective adsorption of DNA was determined using a batchwise method with 0.2 g of the wet adsorbent and 2 ml of a BSA-containing solution.

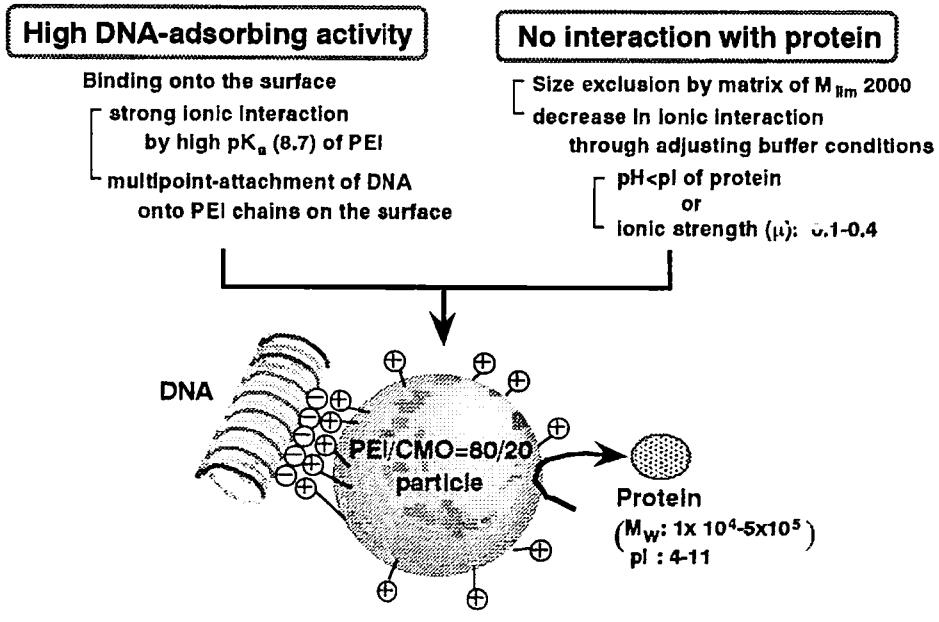

Fig. 5-9. Several factors for the selective removal of DNA from protein solutions by PEI/CMO adsorbents.

5.8. Summery for Chapter 5

The present results suggest that PEI/CMO=80/20 spherical particles can remove any DNA from biologically active substances produced not only by traditional methods but also by recombinant DNA technology. Fig. 5-9 shows several factors for the selective removal of DNA from protein solutions by PEI/CMO particles. The high DNA-adsorbing activity of the PEI/CMO particles is possibly due to (1) strong cationic properties by the higher pK_a of PEI (8.7) than chitosan (6.2), (2) multipoint-attachment of DNA onto the long and flexible PEI chains on the particle's surface. On the other hand, their high DNA selectivity (no interaction with proteins) is due to (1) the size-exclusion effects on protein molecules through their small pore sizes (M_{lim} $2x10^3$), (2) the effect of a decrease in hydrophobic binding which arises when the content of the cross-linked agent (in the particles) is adjusted to 20 mol%, and (3) the effect of the decrease in ionic

interaction which arises when the buffer's pH value or its ionic strength (μ) is adjusted to less than the pI of the protein or to 0.1-0.4, respectively.

For practical applications, ease of regeneration is very important. The cross-linked PEI (PEI/CMO) particles can be completely regenerated by frontal chromatography with 0.2 M sodium hydroxide, followed by 2.0 M sodium chloride. Their stable structures, resisting extreme pH values, are due to their–CHNH- bonds by cross-linking. In addition, we believe that the PEI/CMO particles are better column-packing materials for DNA removing because of their higher flow-rate resistance than that of conventional polysaccharide gels.

5.9. **References for Chapter 5**

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Chapter 6 Conclusion

6.1. PEI-CEIA system to detect Gram-negative bacteria LPS

EIA is a major work force in diagnostic, medical, pharmaceutical, food industrial, environmental and other related researches and investigations. In EIA, antibody and its enzyme conjugate are the most important part but other components for them to support EIA such as blocker against the nonspecific adsorption, stabilizer, enzyme's substrate and matrix are very crucial for successful EIA system to establish.

The ultimate goal was to form a new type of EIA system as a base set to detect food borne hazardous bacteria. It has to be very sensitive, accurate, easy to operate economically, safe for users, producers and environment using safe chemicals and procedures.

Available EIA matrix are polystyrene microwell plate, nitrocellulose, PVDF and others. These matrixes adsorb antibody to capture target antigen. However the matrix adsorbed antibody is not stable enough to store. A new EIA based on target antigen (Gram-negative bacteria LPS) adsorbed onto polymyxin or PEI-polyester cloth and the large adsorption surface and macroporosity permit rapid assays.

The sample preparation is done in extraction buffer which procedure kills bacteria by heating and extracted LPS is dispersed by cholate rather than forming micelle, which in turn enhances sensitivity of the EIA.

Newly developed PEI-cloth and p-cloth can capture LPS specifically. Both cloths are prepared by immersing polyester cloth in PEI or polymyxin solution (physisorption method). It was found that low molecular weight PEI (800) and polymyxin adsorbed to saturation onto cloth in rather low amine contents but higher the molecular weight of PEI, the more amine contents can be adsorbed on the cloth. For CEIA use, it is important to have rather low amine contents of 0.1~0.35 meq/g-cloth which is within the range for blocker to inhibit nonspecific adsorption of antibody. PEI-cloth can be also prepared chemisorption way using water free PEI besides physisorption way prepared as

above. PEI-cloth regardless of the PEI molecular weight, but with the amine group contents of 0.1~0.35 meq/g immobilized either in a physisorption-like or chemisorption-like manner, adsorbed LPS rapidly, preferentially and effectively with minimum of nonspecific adsorption. PEI-cloth is found to be very stable and usable after stored at room temperature even for 7 years. It is advantage for PEI as well that it costs about 100^{th} of polymyxin B.

6.1.1. **Powder TMB substrate reagents**

TMB is a widely used chromogen for HRP based detection systems because its color product has high absorption coefficint, no carcinogenicity and mutagenicity,. However it has poor solubility and stability in aqueous buffers and has to be prepared freshly before each experiment. To overcome these problems, powder form of water soluble TMB was prepared. To solubilize the water insoluble TMB in water, TMB was dissolved in molten PEG 3400 in the ratio of 1 to 250 respectively and the solidified TMB/PEG cake was powdered. This procedure made TMB soluble in water and the mixing with other powder ingredients such as buffer, dextran sulfate and sodium perborate to prepare TMB reagents (soluble and insoluble) did not form any precipitation in water. These powder reagents are found to be quite stable under dry condition and shielded from UV for at least 7 years when stored at room temperature. Other advantage in powder form is that it does not take space nor need to keep in refrigerator for storage and shipping. This makes a field work light and easier.

6.1.2. **Blocker-stabilizer**

Antibody and enzyme conjugate are not stable and form nonspecific adsorption which are part of major problems in EIA. The stabilizer for the conjugate and blocker to prevent the nonspecific adsorption were extracted as one fraction from powder skim milk. The extract is easy to prepare and had strong blocking and stabilizing abilities for CSA-HRP conjugate. The effective blocking enhanced the sensitivity of CEIA because it lowered the background and enhanced the signals. The unusually strong stabilizing effect on CSA-HRP

conjugate by the fraction without loss of activity for one month incubated at 50 ℃ which is equivalent to at least 7 months stored at room temperature. By a gel-filteration column chromatography, the fraction had two major peaks. The first peak is casein which has strong blocking activity and the second peak has strong stabilizing effect and some blocking consisting of small molecular weight of about 1000 possibly oligosaccharides with amine group or peptides. The stabilizer helps to prolong the life of costly conjugate and give consistent results. It enables to transport it at ambient temperature and conduct field test without using cooling facilities.

6.1.3. **CEIA against Conventional EIA**

P-CEIA was developed first by Blais and Yamazaki but had some draw backs such as few availability of polymyxin, high cost, and especially rejection of sample absorption.

The merits of CEIA compared to well-EIA are as follows.

1) The large surface area of the macroporous matrix polyester cloth provides for more rapid and efficient enzymatic and immuno reactions than do non-porous flat microwell

2) The macroporosity of the cloth renders faster washing, pipetting and immuno-concentration of samples than do microporous membranes such as nitrocellulose and PVDF membranes.

3) CEIA can be used for both methods of spectrometric measurements (quantitative) and dot blot EIA (qualitative). None of conventional methods are able to use same medium (matrix) for both EIA.

4) Its sheet form permits simultaneous and rapid processing of multiple samples at same time without need of timed pipettings, equalizing reactions and timed washings.

5) Assay method itself does not require special techniques or skill and simple, and does not need require to use expensive set ups.

6) Sample handling with dead bacteria by heating at 100°C with cholate to extract LPS to do assay is safe for operator and the environment.

6.1.4. **PEI-CEIA**

Newly developed PEI-cloth was examined for suitability for CEIA and compared with p-CEIA. PEI-cloth captured LPS rapidly and reproducibly, and had a detection limit of LPS 10 ng/ml, which was equivalent to ~$1.6x10^5$ cell/ml. Both coated- and covalently-bonded-cloth with PEI-cloth of amine content of 0.1~0.35 were better suited for CEIA than p-cloth.

PEI-CEIA is not only for *Salmonella* LPS detection but applicable in general for other Gram-negative bacteria's LPS (e.g. *E coli* O157:H7, *Campylobactor* etc). PEI-CEIA was found to be easier to handle because the samples can be applied directly on the dry cloth, more economical because PEI cost about 100^{th} of polymyxin and sensitive because PEI-CEIA is 10 to 100 times more sensitive in detection limit and capture LPS 2 times faster than polymyxin-CEIA. The quick reactivity and high sensitivity contribute to shortening assay time and contribute to shorten in total detection time. PEI-cloth preparation is easy with simple low cost equipment. Utilizing the ability in quick capturing *Salmonella* LPS and the macroporous nature of PEI-cloth, rapid detection of a minute amount of *Salmonella* LPS in large volumes by PEI-CEIA was developed.

Either coated or chemically bonded. PEI-cloth is easy to prepare as polymyxin-cloth and PEI is about 100 times less expensive than polymyxin. As polymyxin-cloth was stable enough to be used after 10 years stored at room temperature, the PEI-cloth could be at least as stable as polymyxin-cloth because it has been usable after 7 years preparation. PEI-cloth has an advantage of direct sample application to the dry-state cloth and was able to capture LPS much faster than polymyxin-cloth, eg, PEI cloth within 2 minutes, while polymyxin-cloth took at least 15 min., which may reduce assay time. It was found that PEI 800 bonded-cloth with incubation for 2~5 minutes (low incorporation of PEI) at 110°C gave best sensitivity by dot blot method, and able

to detect a sample (5_ l) of *Salmonella* LPS 10 ng/ml (= ~1.6×10^5 cells/ml) that appeared as a weak but definite blue spot, while coated PEI- coated cloth prepared from more than 2 hr incubations at 40°C gave equivalent sensitivity with polymyxin-cloth detected 100 ng/ml with clear blue spot but no spot was observed below 10 ng/ml. It was also possible to detect *Salmonella* LPS in the presence of non-*Salmonella* LPS in 100 times excess. The quick capturing LPS ability of PEI-cloth and macroporosity of cloth prompted us to develop a method to concentrate a very minute amount of LPS in large volume sample by passing through PEI-cloth cartridge. This method made possible to detect *Salmonella* LPS 1pg in 1 ml sample or 10 pg in 10ml sample. It was possible to detect 1 μg LPS in 10ml chicken soup. We have demonstrated that PEI-cloth is suitable as a specific adsorbent for *Salmonella* LPS and permits the qualitative and quantitative assays in the identifications of *Salmonella* and other Gram-negative bacteria by PEI-CEIA.

6.2. **Removal of unwanted LPS and DNA from medical products by PEI spherical beads**

To remove LPS and nucleic acids from a solution of a cell product used as a drug, water-insoluble poly(ethyleneimine) (PEI) spherical particles were prepared by suspension cross-linking with PEI and chloromethyloxirane. The PEI content of the particles was easily adjusted by changing the PEI ratio and the CMO ratio in the cross-linking. The cross-linked PEI particles, which had diameters of 44 to 105 μm and PEI contents of 50 to 90 unit-mol%, were used as adsorbents. The adsorptions of LPS, DNA and cell products to the adsorbents were determined using a batchwise method. The larger the PEI content of the adsorbent, the larger the LPS and DNA-adsorbing activity of the adsorbent.

For LPS absorption, PEI/CMO=80/20 bead removed more than 99% of 100 ng/ml LPS leaving less than 0.01 ng/ml under physiological conditions (ionic strength of μ= 0.17, pH 7.2). When LPS (100 ng/ml) is contained in protein solutions of BSA, γ-globulin and cytochrome C (1mg/ml each), 99% or more of LPS was removed from each protein solution while 99% of each protein was

retained in the solution. Thus PEI/CMO=80/20 bead is effective in selective removal of LPS from protein solution under physiological conditions.

For DNA removal, the apparent dissociation constant between the DNA (purified DNA from salmon spermary) and the adsorbent decreased from 8.5x 10^{-8} to 9.5 x 10^{-10} M with an increase in PEI ratio from 50 to 90 unit-mol% under physiological conditions (ionic strength of μ= 0.17, pH 7.2). On the other hand, the adsorbing activity of bovine serum albumin also increased with increasing PEI ratio of the adsorbent from 70 unit-mol% or higher, but sharply decreased with increasing ionic strength of the buffer. The adsorbing activity of γ-globulin increased with decreasing PEI ratio to 70 unit-mol% or lower. As a result, when the cross-linked PEI particles, having a PEI ratio of 80 unit-mol%, were used as the adsorbent, they only selectively removed DNA from various protein solutions at an ionic strength of μ= 0.17 and a pH of 7.2. The particles decreased the concentration of DNA in each protein solution to less than 10 ng ml^{-1}, and the recovery rate of protein was more 97% in all cases. For practical applications, ease of regeneration is very important. The cross-linked PEI (PEI/CMO) particles can be completely regenerated by frontal chromatography with 0.2 M sodium hydroxide, followed by 2.0 M sodium chloride. Their stable structures, resisting extreme pH values, are due to their–CHNH- bonds by cross-linking. In addition, we believe that the PEI/CMO particles are better column-packing materials for DNA removing because of their higher flow-rate resistance than that of conventional polysaccharide gels. Highly modified PEI cloth may have good potentiality for removing LPS and DNA from medical fluid using the benefit of the macroporosity. Either PEI-cloth or beads had good selectivity to adsorb LPS and DNA.

It was found that beads cannot be used for EIA because it contained too high amount of PEI than 0.35 meq/g to see the dif of specific adsorption and nonspecific adsorption..

6.3. **Further possible applications and usages of PEI-cloth and spherical beads**

PEI itself has many other functions such as chelating heavy metals, killing bacteria, flocculation and the macro-porosity of cloth, a highly modified PEI-cloth (e.g. amine content >3 meq/g) can be used for:

1) removal of heavy metals and bacteria from drinking water and kill bacteria.

2) recovery of heavy metals from industrial waste water or sea water.

3) removal of LPS, heavy metal and other contaminants like metabolic wastes in blood in circulation to purify, because PEI-cloth gives good flow, stand against pressure and clotting and no leakage but bead form is not suitable for this use because it may clot easily and slow to circulate.

4) use for ion exchange column pack. Small pieces of cloth can be packed in the column which has good flow rate and stand against pressure and good separation of proteins. Also use for preliminary large volume purification of protein or enzyme by batch method.

5) use as air filter and collecting dust cloth because dust and bacteria may be negatively charged and PEI has bacteria killing power.

6) beads can be used for removing LPS and DNA as above studied but could be more effective as being used as 2^{nd} or final remove stage after using PEI-cloth treatment.

7) beads can be used for ion exchange column pack which may give good separation and recovery of proteins, DNA and LPS.

8) beads can be used for recovery of precious metals and other contents from sea water