EFFECT OF A NOVEL SOLID PHASE ON ANTIGEN-ANTIBODY REACTIONS : ITS POSSIBLE APPLICATIONS TO IMMUNOASSAY

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by

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Contents

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Abstract

A new solid-phase constructed from natural rubber (NR) latex was formulated for Hepatitis B surface antigen (HBsAg), anti-Hepatitis B surface antigen (anti-HBs), Hepatitis C virus antibody (anti-HCVAg) and Thyroxine (T4) assays with polypropylene (PP) solid phase as control. The study showed that the percent binding of proteins was much higher on NR than on PP surface. NR film surface was applicable for both sandwich (HBsAg, anti-HBs, anti-HCVAg assays) and competitive binder ligand immunoassays (T4 assay). Experiment on dried NR coated tubes stored at 26°C showed that NR coated tube can be stored for up to at least six months without significant changes in specific binding. In addition both anti-HBs or HBsAg-immobilised NR/PP tubes showed no significant changes in specific and non-specific bindings for both anti-HBs and HBsAg after 3 months of storage at 4°C. However, the sensitivity of anti-HBs, HBsAg, T4 and anti-HCVAg assays was rather low as compared to PP solid phase despite high percent binding of proteins. The optimum coating concentration of HBsAg for PP tube was 0.88 μg/ml, however higher concentration of 1.76 μg/ml was required to saturate the washed natural rubber (WNR) surface. In the case of PP surface immobilised with anti-HBs, the optimal coating concentration was 260 μg/ml. Higher anti-HBs concentration was required to increase further the surface binding of ^{125}I anti-HBs by NR surface immobilised with anti-HBs with a concomitant increase in the non-

specific binding. Therefore concentration of coating solution of 260 μg/ml was used for anti-HBs and 1.76 μg/ml for HBsAg in the subsequent experiments.

The adsorption of protein on both PP and NR surfaces was studied using scanning electron microscopy (SEM) and atomic force microscopy (AFM) techniques. Formation of a thin layer evenly spread out, dendrite-like aggregates of protein was observed on PP surface. However adsorption of protein on NR surface was in cluster form and submerged into the NR coating. These could possibly be due to (i) difference in hydrophobicity of the two surfaces: PP surface was more hydrophobic promoting unfolding of proteins molecules and covering the surface more evenly, whereas the adsorbed antigen-antibody molecules did not unfold on the less hydrophobic NR surface. (ii) NR surface contained inorganic substance and proteins. These may interact with the immobilised protein resulting in the formation of aggregates, thus high percent binding of these on NR surface was not due to the uneven NR surface.

In the assay analysis, the low sensitivity of NR surface can be explained as follows :- (i) Occurrence of substantial denaturation of polyclonal antibodies and antigens passively adsorbed on NR coated surface resulting in a decrease in binding properties of the immobilised antibodies or antigens; (ii) Even coating of proteins on PP surface provides an increased surface area for interaction between serum antigen or antibody with the immobilised macromolecules. On NR surface, the clusters formed were partially embedded in the NR coating resulting in reduced interaction with the antibodies or antigen; (iii) Unfolded antibody or antigen

proteins on PP surface were tightly adsorbed and cannot be displaced with ease by other molecules, whereas on NR surface, added antibody or antigen could penetrate through the NBCS layer (used for blocking the active site of solid phase after coating with anti-HBs or HBsAg). In addition, desorption of immobilised proteins from the NR solid surface occurred as it is not so tightly bound as compared to those on PP surface.

To improve the sensitivity of the assay, the following treatments were carried out on the NR surface :

(i) NR surface was typsinised or washed with HCl, PBS and distilled water prior to immobilisation of antibodies and antigen.

It was found that treatment of NR latex film surface with acid, PBS, water and trypsinisation improved the surface properties. The above treatments did not increase the percent of antibody or antigen adsorbed on the NR surface, but were able to remove non-rubber materials from the NR surface such as rubber proteins which were exuded from the NR film when dried. Longer incubation with trypsin and PBS produced cleaner surface with higher sensitivity because the rubber proteins which were embedded in the interior of the NR film could migrate to the surface and were easily leached out from the NR film surface by the procedure. During trypsinisation, trypsin deposited on the NR surface resulted in the increase of non-specific binding. Moreover, the removal of ammonia during film treatment lowered the pH of the NR film surface resulting in the reduction of the charges of the amino acid in the Nernst's layer of immobilised antigen-antibody proteins. Such

alterations in the charge of specific amino acids may change the binding affinity of the immobilised antigen-antibody complex. In addition, alteration of the pH may change hydrogen bonding, dipole interactions and hydrophobic forces between the structural features. Changes in structural conformation of the bound macromolecules resulting in suitable orientation of the molecules for enhanced antibody-antigen interaction.

(ii) NR surface was precoated with blockers.

Precoating reduced the non-specific binding but its effect on specific binding was dependent on the hydrophobicity of the solid phase. This is due to the low adsorption of immobilised protein (antibody or binder) after the precoating step since the bare surface area of the substrate for adsorption was reduced. Further, the characteristics of the solid phase was changed in the process. The NEO and NBCS blockers contained much higher protein concentration and also mixture of various size proteins and were thus able to adsorb onto hydrophobic solid surface more efficiently. Therefore it prevented anti-HBs or HBsAg to adsorb on to the precoated surface. As a consequence, specific binding was reduced to a greater extent. However precoating with 0.5% of gelatin on the relatively hydrophilic NR surface was able to enhance the sensitivity of HBsAg and T4 assay. For the hydrophobic PP surface, the greater conformational changes of immobilised proteins resulted in greater surface area being covered and stronger binding . Thus they were not easily displaced by the addition of protein such as anti-HBs or HBsAg in the second

immobilisation process. Therefore precoating on PP surface reduced the sensitivity of the assay to a greater extent as compared to NR surface.

In addition, experiments were designed to seek suitable blockers that could reduce non-specific binding. We found that for NR as a solid phase, NEO and NBCS were effective blockers. Both were effective in covering the unbound surface. However high adsorption of the blockers were also accompanied by a high desorption of immobilised proteins (antibodies and binder) which reduced the specific binding. Therefore in choosing a suitable blocker, two factors must be considered: the adsorption of blockers and desorption of immobilised antibody/antigen protein. Gelatin which can cover the unbound surface moderately and does not result in high desorption is considered as a good blocker for NR surface.

Abstrak

Sejenis fasa pepejal baru yang diperbuat daripada lateks getah asli (NR) digunakan untuk mengasai HBsAg, anti-HBs, anti-HCVAg dan T4. Fasa polipropilena (PP) digunakan sebagai kawalan. Keputusan penyelidikan menunjukkan penjerapan protein pada permukaan NR adalah lebih tinggi daripada permukaan PP. Lapisan NR boleh digunakan untuk imuno asai terikat ligand secara 'sandwich' (asai HBsAg. anti-HBs dan anti-HCVAg) dan pertandingan (asai T4). Pengajian penyaduran NR ke atas tabung polipropilena yang disimpan selama enam bulan pada 26°C tidak menunjukkan perubahan ikatan spesifik yang bererti. Tambahan pula, NR serta PP yang dijerap dengan anti-HBs atau HBsAg, untuk tempoh tiga bulan pada suhu 4°C tidak menunjukkan perubahan ketara dalam pengikatan spesifik dan pengikatan tidak spesifik. Kesensitivitian adalah rendah bagi asai anti-HBs, HBsAg, T4 dan anti-HCVAg yang terjerap pada permukaan NR jika dibanding dengan PP sebagai fasa penjerapan, walaupun, penjerapan protein pada NR adalah lebih tinggi. Kepekatan optimum untuk penjerapan HBsAg pada tabung PP ialah 0.88 μg/ml manakala kepekatan yang lebih tinggi iaitu 1.76 μg/ml diperlukan untuk permukaan NR. Kepekatan optimal untuk penjerapan anti-HBs pada permukaan PP ialah 260 μg/ml. Kepekatan yang tinggi diperlukan untuk permukaan NR supaya meningkatkan penjerapan ^{125}I anti-HBs, tetapi peningkatan kepekatan anti-HBs juga mengakibatkan penambahaban ikatan tidak spesifik.

Dengan itu, kepekatan anti-HBs dan HBsAg yang diguna bagi semua eksperimen ialah 260 μg/ml dan 1.76 μg/ml masing-masing.

Teknik SEM dan AFM telah digunakan untuk pengajian yang terperinci bagi penjerapan protein pada permukaan PP dan NR. Lapisan nipis anti-HBs/ HBsAg berbentuk 'dendrites' diperhatikan pada permukaan PP. Pada permukaan NR pula, lapisan protein adalah berbentuk ketulan ('aggregates/clusters'). Perbezaan rupabentuk tersebut boleh dijelaskan seperti berikut.

(i) Perbezaan kehidrofobikan NR dan PP

Permukaan PP adalah lebih hidrofobik. Sifat kehidrofobikan ini mengizinkan penolakan protein dan menyebabkan lapisan molekul terdedah. Permukaan NR yang kurang hidrofobik, tidak menggalakkan pembukaan molekul antigen/antibodi.

(ii) NR mengandungi bahan bahan inorganik dan protein.

Bahan-bahan inorganik ini berkemungkinan bertindakbalas dengan antibodi/antigen yang terjerap pada permukaan dan membentuk 'aggregates'. Peratusan penjerapan yang tinggi pada permukaan NR mungkin disebabkan oleh pembentukan 'agregates' tersebut.

Kesensitivitian yang rendah pada permukaan NR bagi imunoasai boleh diterangkan seperti berikut.

(i) Antibodi poliklonal dan antigen yang terjerap pada permukaan NR mungkin denaturasi. Dengan ini, menurunkan kereaktivitian meraka.

(ii) Antibodi/antigen yang terjerap pada permukaan PP membentukkan lapisan yang rata serta nipis. Dengan ini meningkatkan interaksi ianya dengan serum antigen /antibodi. Sebaliknya ketulan antibodi/antigen yang terhempit pada NR mengurangkan interaksi antara antibodi dan antigen.

(iii) Pendedahan molekul protein pada permukaan PP dapat menjerap dengan lebih kuat, dengan itu tidak mudah tersingkir oleh molekul lain semasa pengasaian. Pada permukaan NR pula, keadaan adalah sebaliknya. Di samping itu, protein yang ditambah semasa pengasaian mungkin menembusi lapisan NBCS (ia digunakan untuk menutupi bahagian pepejal yang tidak terjerap dengan anti-HBs & HBsAg.).

Untuk meningkatkan kesensitivitian asai, beberapa modifikasi kepada lapisan NR telah dijalankan.

(i). Permukaan NR telah dibilas dengan HCl, PBS, air serta diliputi dengan tripsin sebelum penjerapan antigen & antibodi.

Keputusan menunjukkan keupayaan permukaan diperbaiki di mana bahan-bahan bukan getah seperti protein telah disingkirkan daripada permukaan NR dengan proses pembilasan tersebut. Walau bagaimanapun, peratusan penjerapan antibodi/antigen pada permukaan NR tidak menunjukkan peningkatan akibat proses ini. Pembilasan tersebut dapat menyingkir dan mengeluarkan bahan-bahan bukan getah di permukaan NR. Pemanjangan masa eraman dengan tripsin dan PBS, menghasilkan permukaan yang lebih bersih serta lebih sensitif . Ini boleh diterangkan bahawa protein yang terhempit pada lapisan NR boleh bergerak ke permukaan luar dan senang dibilas keluar dengan proses tersebut. Semasa proses

tripsinasi, tripsin terjerap pada permukaan NR dan mengakibatkan ikatan tidak spesifik. Di samping itu, ammonia yang tersingkir semasa pembilasan menurunkan pH lapisan NR dan mengakibatkan penurunan cas asid amino pada lapisan Nernst yang terjerap dengan antigen dan antibodi. Perubahan cas pada asid amino mungkin menyebabkan keafiniti penjerapan antibodi/antigen. Tambahan pula, perubahan pH mengubah kekuatan ikatan hidrogen, interaksi dwikutub dan daya dwikutub antara struktur molekul. Seterusnya mengubah konformasi makromolekul yang mengakibatkan orientasi molekul yang lebih sesuai untuk penjerapan antibodi/antigen.

(ii) Permukaan NR telah dijerap dengan 'blockers'

Penjerapan 'blocker' dapat menurunkan ikatan tidak spesifik. Kesannya ke atas ikatan spesifik bergantung kepada kehidrofobikan fasa pepejal. Pemerhatian ini boleh diterangkan bahawa, selepas 'precoating', keupayaan penjerapan antibodi/antigen turun akibat daripada luas permukaan substrate dikurangkan. Di samping, ciri-ciri fasa pepejal ini turut terubah dalam proses ini. Kepekatan protein yang tinggi serta pelbagai saiz dari NEO dan NBCS memperbolehkan ianya menjerap pada fasa pepejal dengan lebih berkesan. Maka penjerapan anti-HBs atau HBsAg pada permukaan 'precoated' terhalang. Seterusnya, ikatan spesifik dikurangkan. Walau bagaimanapun, pada permukaan NR yang lebih hidrofilik, 'precoat' dengan 0.5% gelatin boleh meningkatkan kesensitivitian asai HBsAg dan T4. Bagi permukaan hidrofobik PP, perubahan konformasi pada protein terjerap

lebih ketara. Dengan itu, kebanyakan permukaan menjadi tertutup dan ikatan pula lebih kuat. Lantaran, mereka sukar digantikan oleh peotein seperti anti-HBs atau HBsAg dalam proses penjerapan kedua. Secara kesimpulan, 'precoating' pada permukaan PP mengurangkan kesensitivitian asai dengan lebih berkesan jika dibanding dengan permukaan NR.

Penyelidikan juga dijalankan demi mendapat 'blocker' yang sesuai yang boleh mengurangkan ikatan tidak spesifik. Keputusan menunjukkan sekiranya NR adalah fasa pepejal, NEO dan NBCS merupakan 'bloker' yang efektif. Kedua-dua ini berkesan dalam menutupi bahagian permukaan yang tidak dijerap. Keupayaan penjerapan 'blocker' yang tinggi diikuti dengan penyingkiran protein yang terjerap, akibatnya ikatan spesifik dikurangkan. Maka, semasa memilih 'blocker' yang sesuai, dua faktor perlu dipertimbangkan : iaitu penjerapan 'blocker' dan penyingkiran protein yang terjerap. Gelatin boleh menutupi permukaan yang tidak terjerap tanpa menyebabkan penyingkiran protein dan ianya merupakan 'blocker' yang baik untuk permukaan NR.

Term	Abbreviation
Anti-Thyroxine	anti-T4
antibody immunoradiometric assay	IRMA
Antibody/ Antigen	AA
Atomic force microscopy	AFM
Bovine IgG	BIgG
Bovine serum albumin	BSA
Capture antibody	Cab
Deprotenised natural rubber latex	DPNR
Electron dense core of HBsAg	HBcAg
Fetal calf serum, newborn	NBCS
Fourier transform infra-red spectroscopy	FTIR
Hepatitis C virus	HCVAg
Hepatitis e antigen	HBeAg
Horse Hepatitis B Antibody	Anti-HBs
Hepatitis B Antigen	HBsAg
High ammonia NR latex	HA
Human IgG	HIgG
Human serum albumin	HSA
Immunoglobulin	Ig
Institute of Atomic Energy Beijing China	CIAE
Lactalbumin	LA
Monoclonal antibody	Mab
Natural rubber	NR
Neo natal serum	NEO
Non specific binding	NSB
Non specific binding with serum preincubation	NSB(S)
Non specific binding without serum preincubation	NSB(WS)
Phosphate buffer saline	PBS
Poly (2-hydroxyethyl acrylate)	PHEA
Polyacrylic acid	PAA
Polyehtyleneterephthalate	PET
Polyethylene oxide	PEO
Polymethacrylic acid	PMAA
Polypropylene oxide	PEO
Polypropylene tube	PP
Polystyrene	PS
Polyvinyl toluene	PVT
Polyvinylchloride	PVC
Rabbit IgG	RIgG
Radioimmunoassay	RIA
Scanning electron microscopy	SEM
Specific binding	SP

Term	Abbreviation
Synthetic isoprene latexs	SL
Thyroxine	T4
Thyroxine standards	T4 Std
Total Internal reflection flourescence	TIRF
Transmission electron microscopy	TEM
Washed natural rubber surface	WNR