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A simple, rapid and inexpensive technique to bind small peptides to polystyrene surfaces for immunoenzymatic assays

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Abbreviations: DCLM, Direct Cross-linking Method. ICS: In-Solution Coupling.

Abstract

Synthetic peptides are widely used in indirect ELISA to detect and characterize specific antibodies in biological samples. Small peptides are not efficiently immobilized on plastic surfaces by simple adsorption, and the conjugation to carrier proteins with different binding techniques is the method of choice. Common techniques to conjugate peptide antigens to carrier proteins and to subsequently purify such complexes are time consuming, expensive, and occasionally abrogate immunogenicity of peptides. In this report we describe a simple, fast and inexpensive alternative protocol to immobilize synthetic peptides to plastic surfaces for standard ELISA. The technique is based on use of maleimide-activated Bovine Serum Albumin or Keyhole Limpet Hemocyanin as a protein anchor adsorbed on the polystyrene surface of the microtiter plate. Following adsorption of the carrier protein, sulfhydryl-containing peptides are cross-linked with an in-well reaction, allowing their correct orientation and availability to antibody binding, avoiding the time consuming steps needed to purify the hapten-carrier complexes. The immunoreactivity of peptides was tested by using both monoclonal and polyclonal antibodies in standard ELISA assays, and compared with established coating methods.

Keywords: ELISA; Synthetic peptides; coating procedures.

Introduction

Synthetic peptides are widely used in epitope mapping and as immunogens for antibody production and detection. In order to be used as antigens in immunological assays such as ELISA, peptides need to be immobilized on the plastic surface of multiwell plates. The adsorption of antigens involves non-covalent bonds between the hydrophobic regions of immunogenic proteins and the non-polar polystyrene surface. Although proteins are readily and efficiently adsorbed, peptides shorter than 15 residues are not efficiently immobilized on surfaces by simple adsorption and may be partially washed away during standard ELISA procedures (Pyun et al., 1997). In addition, because of their small size, peptides often bind to the plastic substrate with an orientation which may cause the masking of antigenic epitopes, that are therefore not easily accessible to antibody binding. In an attempt to overcome these problems, different methods have been proposed to immobilize peptides to polystyrene surfaces. Plates can be activated with UV or gamma ray irradiation and then treated with glutaraldehyde before plating peptides, that can bind to the plate through primary amino groups reaction (Boudet et al., 1991). Alternatively, peptides can be cross-linked as haptens on a carrier, such as bovine serum albumin (BSA) or polylysine (Price et al., 1993; Ball et al., 1994) and then immobilized on untreated plastic surfaces. Initially, carrier/hapten complexes were cross-linked through carboxyl- or amino groups by using N-hydroxysuccinimide/dicyclohexylcarbodiimide, glutaraldehyde or bis-diazotized benzidine, which require harsh reaction conditions. These reagents may partially or completely abolish the antigenicity of peptides (Briand et al., 1985), especially when Asp, Glu, or Lys residues are present. More recently, covalent coupling of synthetic peptides to Maleimide Activated BSA has been proposed and used with success (Yu et al., 1996). Peptides coupled with this method show a known and predictable orientation, which allows a free interaction with antibodies both in vivo and in vitro (Adrian, 2002). Nevertheless, carrier/hapten complex purification represents a major limit of all coupling protocols. In particular, procedures for purification of cross-linked haptens based on dialysis or gel filtration chromatography are time consuming and expensive.

In order to overcome critical issues of small peptides immobilization to microwell plates, we set up a simple, rapid and innovative method based on a direct in-well coupling of peptide molecules on plates pre-coated with maleimide-activated carriers (BSA or KLH), and we named it Direct Cross-Linking Method (DCLM).

We used peptides designed to display either a N or C terminal cysteine residue for maleimide-mediated conjugation with carrier proteins. In these conditions peptides and carrier protein form stable thioether bonds, and allow for an optimal orientation of epitopes for subsequent antibody recognition. We produced a panel of

polyclonal and monoclonal antibodies against selected peptides and used them to validate and optimize the DCLM and to compare it to established antigen immobilization protocols routinely used in ELISA.

2. Materials and methods

2.1 Synthetic peptides and in-solution peptide-carrier coupling (ISC).

Nine synthetic peptides with length ranging from 10 to 15 aminoacids, were designed to display different hydrophobic/hydrophilic characteristics (Table 1) and to carry either a N or C terminal cysteine, to allow cross-linking with maleimide-activated carrier proteins. All peptides were purchased from Nurex Srl (Sassari, Italy). Conventional in-solution maleimide-mediated coupling reaction (ISC) was performed by using the Inject Maleimide Activated Immunogen Conjugation kit (Pierce, Rockford, IL, USA), according to the manufacturer suggestions. Briefly, 5 mg of each peptide were dissolved in 500 μ l maleimide conjugation buffer (83 mM sodium phosphate buffer, 0.1M EDTA, 0.9 M NaCl, 0.02% sodium azide, pH 7.2) and then coupled to 2 mg of both maleimide-activated bovine serum albumine (BSA-M) and keyhole limpet hemocyanin (KLH-M) by incubating the solution at room temperature for 2 hours. Peptide-carrier proteins were then purified using the gel filtration purification columns provided with the commercial kit.

2.2 Direct Cross-Linking Method (DCLM) and determination of optimal concentration of carrier and peptides

Direct cross-linking of peptides was set up as follows: different amounts of either BSA-M or KLH-M, ranging from 4 μ g to 0,1 μ g per well, were dispensed into microwell plates and incubated at 4°C overnight. Plates were then extensively washed in distilled water. Four peptides (TvExt-1, TvExt-2, TvCt-1, or TvCt-2) were dissolved in maleimide conjugation buffer, serially diluted at a 1:2 ratio to a final concentration ranging from 3 mg/ml to 1,5 μ g/ml and 100 μ l of each dilution was then dispensed into either BSA-M or KHL-M precoated plates. Polystyrene plates were incubated for 2 hours at room temperature to allow cross-linking, then washed with water and ELISA was performed by testing the corresponding polyclonal antisera. Each condition was tested in triplicate.

2.3 Comparison of the DCLM and passive coating with ISC conjugates.

Sensitivities and specificities of direct cross-linking method and of passive-coating method were compared by using polyclonal antibodies anti-TvExt-1, anti-TvExt-2, anti-TvCt-1, anti-TvCt-2 (Noel et al., 2010). Microtiter plates either prepared with DCLM or passively coated with ISC conjugates were washed four times with PBS containing 0.05% Tween 20 (PBS-T). Specific sera were tested at 1:200, 1:500, and 1:1000 dilutions. After 2 hours of incubation at room temperature, plates were extensively washed with PBS-T and binding of antibodies was revealed with a alkaline phosphatase-conjugated anti-mouse polyvalent antibody

(Sigma-Aldrich, Milano, Italy). After thorough washing with PBS-T, colorimetric reaction was developed for 20 minutes using p-nitrophenyl phosphate (Sigma-Aldrich, Milano, Italy). Absorbances at 405 nm were read with a VersaMax instrument (Molecular Devices) and data processed with the SoftMaxPro software. To rule out cross-reactivity, all sera were tested with either unconjugated peptides or BSA-M and KLH-M alone. A pre-immune mouse serum was used as a control. All experiments were performed in triplicate.

2.4 Detection of directly cross-linked peptides by monoclonal antibodies

Since ELISA techniques are frequently used to test the reactivity of anti-peptide monoclonal antibodies (MAb), we produced five murine monoclonal antibodies specific for the synthetic peptides BRCA2, TvSaplip5, TvSaplip6, TvSaplip10 and Tvsaplip12 (see table 1). Briefly, five BALB/c mice were stimulated three times by subcutaneous and intraperitoneal inoculation of 30 µg of KHL-M conjugated peptides in complete Freund's adjuvant at 10 days intervals. Four days after a last intravenous booster with 10 µg of BSA-M conjugated peptide, mice were sacrificed and spleen cells were hybridized with myeloma cell line P3X63.Ag8.653. After selection, supernatants were screened by ELISA to isolate positive hybridomas. Microwell plates were coated with peptides conjugated to carrier proteins with either method (DCLM or ISC).

3. Results and discussion

Peptide cross-linking to plastic surfaces is a well known critical step in ELISA. A number of protocols have been proposed so far, including direct covalent coupling of peptides to microtitre plates, conjugation of peptides to carrier protein (Adrian, 2002), passive binding to multimeric peptides (Tam and Zavala, 1989), and peptide binding to supporting resins (Kennedy et al., 1987). Although these methods lead to an efficient immobilization of peptide antigens, they are expensive, time consuming and may in some cases reduce the antigenicity of peptides (Adrian, 2002).

In the present study we propose a simple and rapid protocol for the direct cross-linking of small peptides to ELISA plates. The polystyrene surface is pre-coated with maleimide-activated BSA or KLH, and peptides are added directly to the wells of microtitre plates for subsequent coupling.

The efficiency of DCLM was tested with four different peptides (TvExt-1, TvExt-2, TvCt-1, or TvCt-2) and subsequent standard indirect ELISA with polyclonal antibodies was performed. All the sera specifically recognized the corresponding specific antigens when directly cross-linked onto the multiwell plate. Results in terms of signal strength were comparable to that obtained with ELISAs performed by using plates coated with ISC conjugates. In two cases (peptides TvCt-2 and TvExt-2), DCLM led to OD values which were higher than that obtained by pre-coating conjugation (Figure 1). No positive reaction was detected by using BSA-M, KLH-M or peptides alone as control antigens.

We assessed the optimal amount of carrier protein to be used in DCLM by adsorbing on a 96-well microplate different BSA-M and KLH-M amounts, ranging from 0.1 µg/well to 4 µg/well. The optimal conditions were 0.5 µg/well and 1 µg/well for BSA-M and KLH-M, respectively. Furthermore, we showed that the optimal peptide amount is 0.5 µg/well for both BSA-M and KLH-M precoated plates. These concentrations are consistent with the optimal molar ratio routinely used in peptide/maleimide-activated carrier coupling performed in solution with commercial kits.

ELISA is widely used also to test the reactivity of monoclonal antibodies. In this application, an optimal epitope exposure is fundamental. DCLM was used to test a panel of monoclonal antibodies, showing to be as efficient as the pre-coating conjugation method. Each monoclonal antibody specifically recognized its peptide antigen when directly cross-linked to either BSA-M (or KLH-M). Price and colleagues proposed an in-situ method based on glutaraldehyde cross-linking (Price et al., 1993), which allows to avoid the

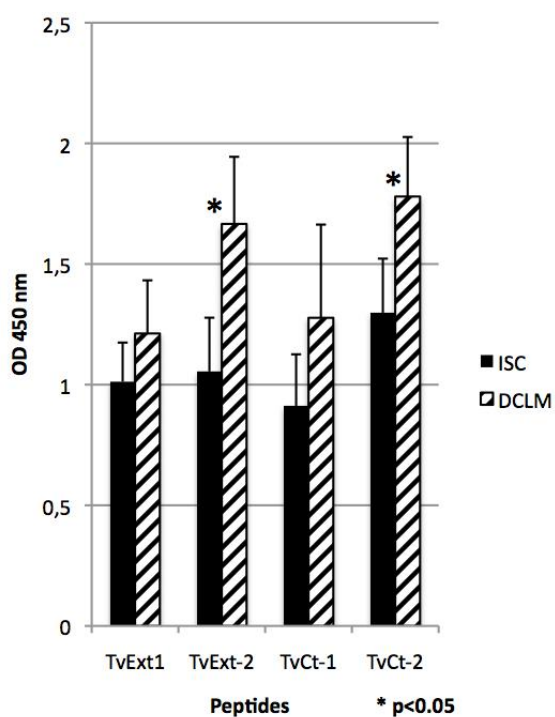
purification step after hapten-carrier conjugation. While being more rapid than conventional methods, this protocol shows the typical pitfalls of glutaraldehyde-based coupling chemistry (harsh reaction conditions, possible diminished immunogenicity) (Adrian, 2002). On the contrary, DCLM combines very efficiently the advantages of both maleimide-activated carriers and direct coupling of peptides to polystyrene surfaces, allowing to avoid the time-consuming and expensive steps of hapten-carrier complex purification. DCLM has proven to be simple, reproducible, cost-effective and suitable with peptides of different lengths and hydrophobic/hydrophilic characteristics.

Altogether, DCLM can be considered a simpler, faster and much more convenient alternative to standard coupling protocols, allowing to save time and cut costs in ELISA applications. When considering its time- and cost-effectiveness, DCLM may prove to be applicable also to automated systems for high-throughput peptide screenings

Table 1

Peptide	Sequence	Molecular mass (Da)
BRCA2	H-HLQSSVVVSDC-OH	1173,29
TvSaplip-5	H-EKGLERGIEEC-OH	1262,38
TvSaplip-6	H-SFKGECQVCKFC-OH	1377,59
TvSaplip-10	H-SDKEISREFNC-OH	1327,41
TvSaplip-12	H-EGEIIIEETTEC-OH	1251,52
TvCt1	H-CPKNKNDDNEGNQED-OH	1719,69
TvCt2	H-CDEQPNNESDSNGLD-OH	1635,60
TvExt1	H-CPKSDSNSMIRLQEI-OH	1720,96
TvExt2	H-CTHKQSEENPNQPGE-OH	1696,71

Figure 1.



Legends to figures

Table 1 Table 1 Aminoacidic sequences of synthetic peptides used in this study. All peptides were designed with either a N-terminal or C- terminal cysteine residue.

Figure 1

Comparison of DCLM and ISC methods. The graph shows results from a representative experiment. Polyclonal antisera were tested at a 1:500 dilution. Bars represent mean values $\pm$ SD.

REFERENCES

- Adrian, T.E. 2002 Production of antisera using peptide conjugates. In: J.M. Walker (Ed.). Humana Press, p. 953-961.
- Ball, J.M., Henry, N.L., Montelaro, R.C. and Newman, M.J., 1994, A versatile synthetic peptide-based ELISA for identifying antibody epitopes. *J Immunol Methods* 171, 37-44.
- Boudet, F., Theze, J. and Zouali, M., 1991, UV-treated polystyrene microtitre plates for use in an ELISA to measure antibodies against synthetic peptides. *J Immunol Methods* 142, 73-82.
- Briand, J.P., Muller, S. and Van Regenmortel, M.H., 1985, Synthetic peptides as antigens: pitfalls of conjugation methods. *J Immunol Methods* 78, 59-69.
- Kennedy, R.C., Dreesman, G.R., Chanh, T.C., Boswell, R.N., Allan, J.S., Lee, T.H., Essex, M., Sparrow, J.T., Ho, D.D. and Kanda, P., 1987, Use of a resin-bound synthetic peptide for identifying a neutralizing antigenic determinant associated with the human immunodeficiency virus envelope. *Journal of Biological Chemistry* 262, 5769-5774.
- Noel, C.J., Diaz, N., Sicheritz-Ponten, T., Safarikova, L., Tachezy, J., Tang, P., Fiori, P.L. and Hirt, R.P., 2010, *Trichomonas vaginalis* vast BspA-like gene family: evidence for functional diversity from structural organisation and transcriptomics. *BMC Genomics* 11, 99.
- Price, M.R., Sekowski, M., Hooi, D.S., Durrant, L.G., Hudecz, F. and Tendler, S.J., 1993, Measurement of antibody binding to antigenic peptides conjugated in situ to albumin-coated microtitre plates. *J Immunol Methods* 159, 277-81.
- Pyun, J.C., Cheong, M.Y., Park, S.H., Kim, H.Y. and Park, J.S., 1997, Modification of short peptides using epsilon-aminocaproic acid for improved coating efficiency in indirect enzyme-linked immunosorbent assays (ELISA). *J Immunol Methods* 208, 141-9.
- Tam, J.P. and Zavala, F., 1989, Multiple antigen peptide. A novel approach to increase detection sensitivity of synthetic peptides in solid-phase immunoassays. *J Immunol Methods* 124, 53-61.
- Yu, Z., Carter, J.M., Huang, S.Y., Lackland, H., Sigal, L.H. and Stein, S., 1996, Presentation of peptide antigens as albumin conjugates for use in detection of serum antibodies by enzyme-linked immunosorbent assay. *Bioconjug Chem* 7, 338-42.