Coupling of peptides to Thermo Scientific Nunc CovaLink Surfaces via their carboxylic groups

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Key Words
Nunc™ CovaLink™ Surface, Covalent attachment, ELISA, Peptides, Protocol.

Goal

The goal of this protocol is to outline the specific steps which are necessary in order to be successful when using the CovaLink surface for binding of peptides.

Introduction

Formation of amide bonds between carboxylic acids and amines is generated by 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) which activates the carboxylate by forming an O-acylurea. However, when the reaction is carried out in an aqueous solution the compound is subject to hydrolysis which can significantly limit the yield. It has been demonstrated that a more hydrolysis resistant active ester can be made by adding Sulfo-N-hydroxysuccinimide (Sulfo-NHS). The O-acylurea activated ester will react with Sulfo-NHS, forming a more stable succinimidyl activated ester (Staros, 1986), e.g. an activated peptide (See Fig. 1).

Preparation of reagents and buffers

Material

Solid Phase: Thermo Scientific Nunc CovaLink NH, Thermo Scientific Nunc MaxiSorp, Thermo Scientific Nunc PolySorp

Dinitrophenyl-Peptide
(DNP-Pro-Leu-Gly) Serva - Cat. no. 52264

Dimethylsulfoxide (DMSO) Merck - Cat. no. 102931

1-Ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDC) Sigma - Cat. no E-7750

Sulfo-N-hydroxysuccinimide
(sulfo-NHS) Pierce - Cat. no 24510

Rabbit anti-DNP antibody horseradish peroxidase conjugate (Ra DNP-HRP) Dako - Cat. no. P402

Ortho-phenylenediamine dihydrochloride (OPD)
Fluka - Cat. no. 78440

Hydrogen Peroxide (H_2O_2), 30% Merck - Cat. no. 107209

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DNP-peptide stock solution

DNP-peptide	14.4 mg
Distilled water	0. 4 mL
DMSO	0. 6 mL

DNP-peptide/NHS solution

DNP-peptide stock solution	0.5 mL
Sulfo-NHS	1.84 mg
Distilled water	ad 10 mL

Note: Use fresh solution

EDC solution

EDC	12.3 mg
Distilled water	ad 10 mL

Note: Use fresh solution

Conjugate Solution

Ra DNP-HRP in CovaBuffer	2.6 µg/mL

Note: Use fresh solution.

Substrate solution

OPD	60 mg
H_2O_2 (30%)	50 µL
Citrate-phosphate buffer	ad 100 mL

Note: Use fresh solution and keep dark.

Phosphate Buffered Saline (PBS)

0.15 M, pH 7.2

NaCl	8.0 g
KCl	0.20 g
$Na_2HPO_4 \cdot 2H_20$	1.15 g
KH_2PO_4	0.20 g
Distilled water	ad 1000 mL

Adjust to pH 7.2 with HCl/NaOH

Citrate-Phosphate buffer

0.1 M, pH 5.0

Citric Acid, H_20	7.30 g
$Na_2HPO_4 \cdot 2H_20$	11.86 g
Distilled water	ad 1000 mL

Adjust to pH 5.0 with HCl/NaOH

CovaBuffer

NaCl	116.90 g
$MgSO_4 \cdot 7\ H_20$	10.00 g
Tween 20	0.50 mL
PBS	ad 1000 mL

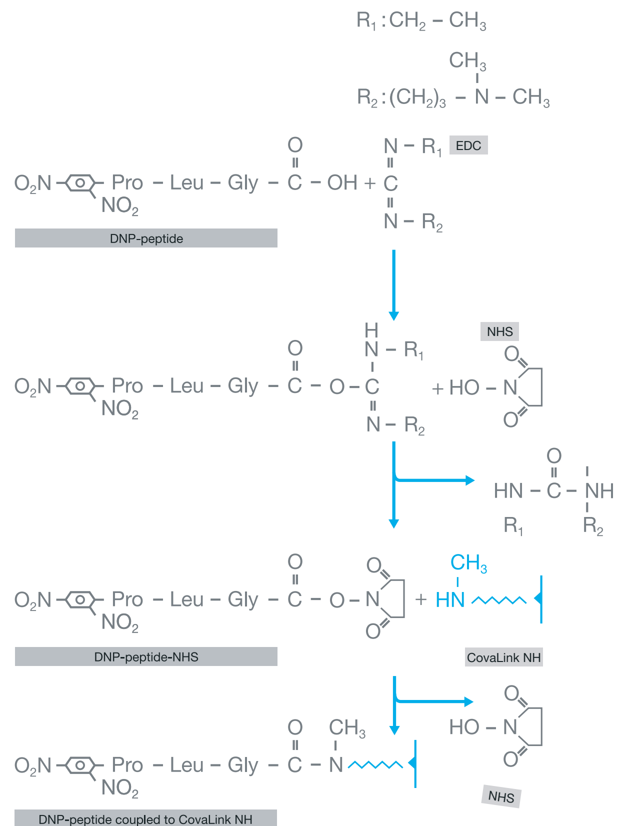

Fig. 1
Reaction scheme for immobilization of DNP-labelled tri-peptide. See text for further information.

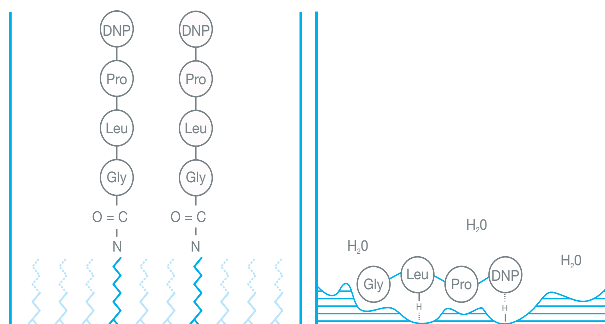

Fig. 2.
Covalent binding of peptide to CovaLink NH (left) and physical adsorption of peptide to MaxiSorp (right).

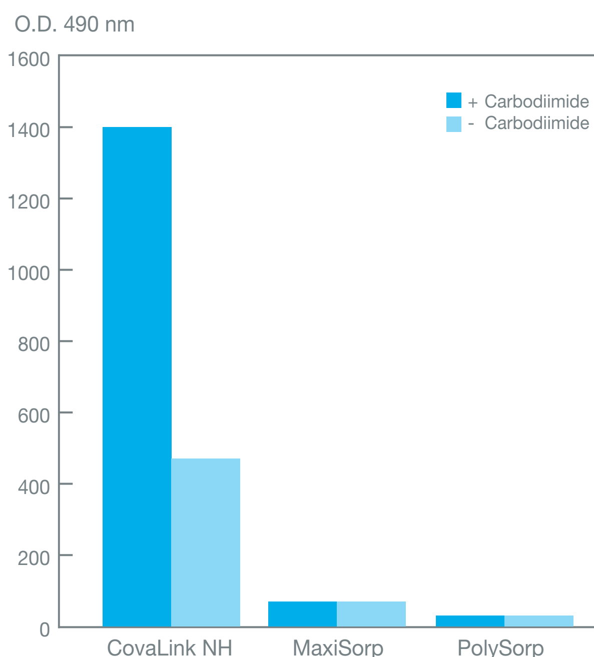

Fig. 3
Illustrates the difference in binding on the three surfaces tested. The observed difference can be explained if the size of the peptide is considered. (See Fig. 2). On the CovaLink NH the small peptide is bound via the carboxylic group to the secondary amino group. On MaxiSorp or PolySorp either the peptide does not adsorb or the molecule is adsorbed but the antigen determinant is undetectable probably due to its inaccessibility to the antibody.

Example

The purpose of this experiment was to demonstrate that a tri-peptide, barely detectable on Nunc™ MaxiSorp™ and Nunc™ PolySorp™, can be detected when bound to CovaLink NH. A tri-peptide, Pro-Leu-Gly, which has only terminal amino and carboxylic groups was used. The terminal proline amino group was labelled with dinitrophenol (DNP), partly to avoid peptide interlinkage, and partly to allow peptide detection by anti-DNP antibody (See Fig. 2).

A. Incubation

Prepare three plates, A. CovaLink, B. MaxiSorp and C. PolySorp as follows

Add 100 ul DNP-peptide/NHS-solution to each well in colum 2.

Add 50 µL distilled water to all other wells.

Prepare dilution series by transferring 50 µL from the wells in column 2 to column 3, mix, transfer 50 ul from 50 µL from column 3 to column 4, mix, etc. After mixing discard 50 µL from the wells in column 12.

Start reaction by adding 50 µL EDC solution to all wells. Cover the plates

Incubate for 2 hours at room temperature.

B. Wash

Empty the wells and wash 3 times with CovaBuffer.

Keep the buffer in the wells for 15 minutes after the third wash.

C. Conjugate Incubation

Empty the wells

Add 100 µL conjugate solution to each well Incubate for 1 hour at room temperature.

D. Wash

As in B above.

E. Substrate Reaction

Empty wells

Add substrate solution, 100 µL /well.

Wait for colour development. To stop the reaction add 1M H_2SO_4 100 µL /well.

Read O.D. of wells at 490 nm.

F. Results

On the CovaLink NH a significant increase in signal was observed by adding carbodiimide, indicating that covalent binding took place. The presence of carbodiimide on MaxiSorp or PolySorp has no effect.

It is interesting to note the level of binding of the peptide on CovaLink NH in the absence of carbodiimide (See Fig. 3). This increase can be explained by the passive adsorption of the peptide on the linker arms of the surface.

G. Conclusion

From this example it can be seen that CovaLink NH can be recommended for the immobilisation of small peptides without the use of a carrier in place of MaxiSorp or PolySorp.

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