AMINATED SURFACE

Surfaces with primary amino groups covalently bound are dedicated to promote the covalent immobilization of compounds containing reactive moieties such as amino, carboxyl or thiol groups via well-known homo-heterobifunctional linkers, e.g. N-Hydroxysuccinimide (NHS) or Succinimidyl 4-(N-maleidomethyl) cyclohexane-1-carboxylate (SMCC).

This kind of immobilization can overcome some of the limitations connected with physical adsorption of the molecules to the surfaces such as:

- immobilization of molecules which are bound weakly or not at all by physical adsorption, namely small peptides (M.W. 1000-5000) drugs, toxins or hormones
- oriented immobilization of molecules in order to secure the integrity and accessibility of their specific sites avoiding the risk of inhibition of these sites by casual physical adsorption for such molecules as Fab-SH-antibody fragments, streptavidin, polysaccharides or nucleic acids (single strand or double strand)
- increased storage stability compared with that of physical adsorption because of the reduced risk of spontaneous desorption

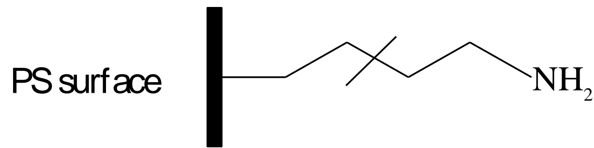
TECHNICAL NOTE N. 6

Introduction to the preparation and use of aminated surfaces for immunological assays

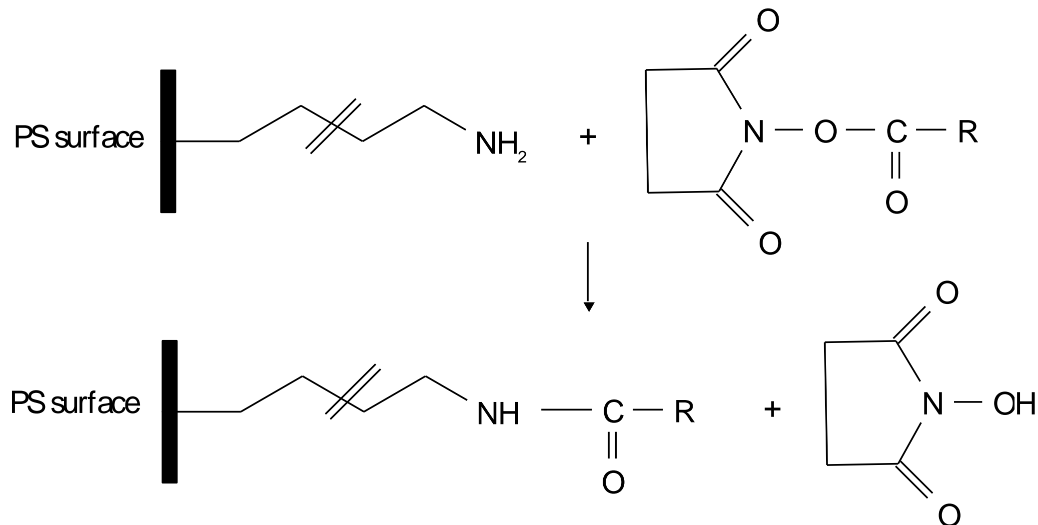
Direction for use

Several recipes are routinely used for the coupling of biological molecules to amino groups. Specific directions for use require the knowledge of the intended application. As general guideline, the interaction between the amino group on the surface and the functional group of the molecule to be bound is based on covalent binding mediated by homo and heterofunctional crosslinkers.
In particular, Ethyldiethylaminopropylcarbodiimide (EDC), with or without the addition of N-hydroxysuccinimide, is a powerful coupling agent of the carboxylic group of the molecule with the amino group of the surface.
If the biomolecule to be bound contains ε amino groups of lysine, the simplest method is coupling via Glutaraldehyde, with the formation of a stable amine linkage by reduction with Sodium Cyanoborohydride.
Other crosslinkers for this purpose are Dimethylpimelidate and Dissucinimidyl suberate.
Biomolecules containing thiolic groups, as Fab-SH or peptides with cystein at terminal end, can exploit the large number of maleimido groups containing crosslinkers as Succinimidyl 4-(N-maleimidomethyl)cycloexane-1-carboxylate (SMCC) for reacting with the amino group.

Schematic chemical and physical configuration of Biomat NH_2 surface



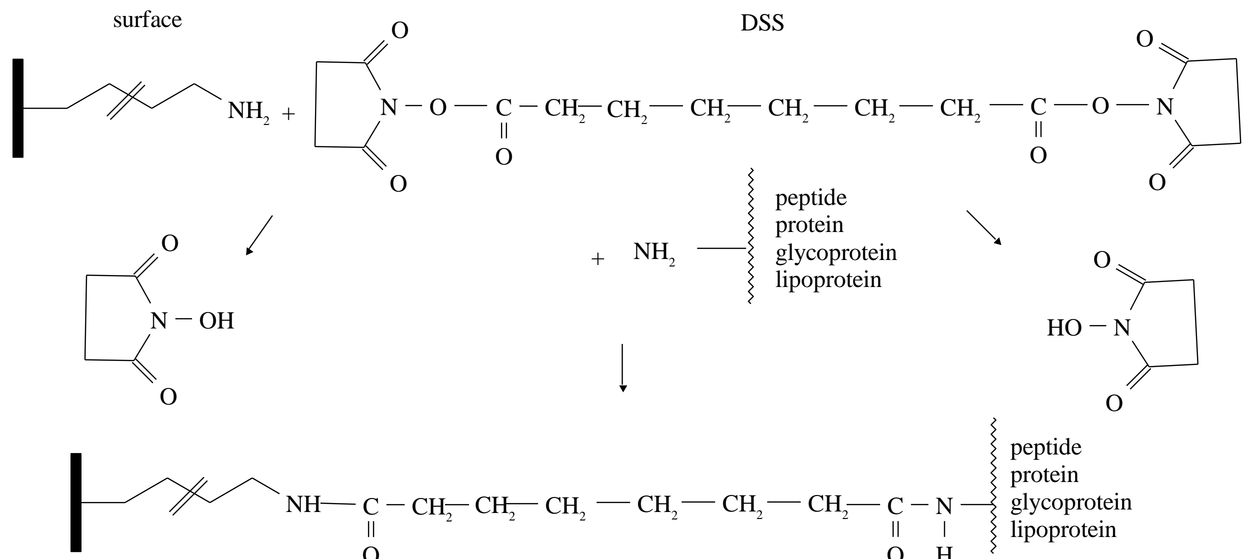
example of reaction scheme:
an arbitrary NHS estherified compound (R) covalently combines with Biomat NH_2 surface through NHS splitting off



Hereunder are some examples of coupling agents to be used for covalent coating of the Biomat NH_2 surface with reactive groups

A. Disuccinimidyl suberate (DSS).
This symmetric (homobifunctional) linker is capable of linking compounds containing secondary or primary amino groups, and can thus be used for covalent immobilisation of peptides, proteins, glycoproteins, lipoproteins

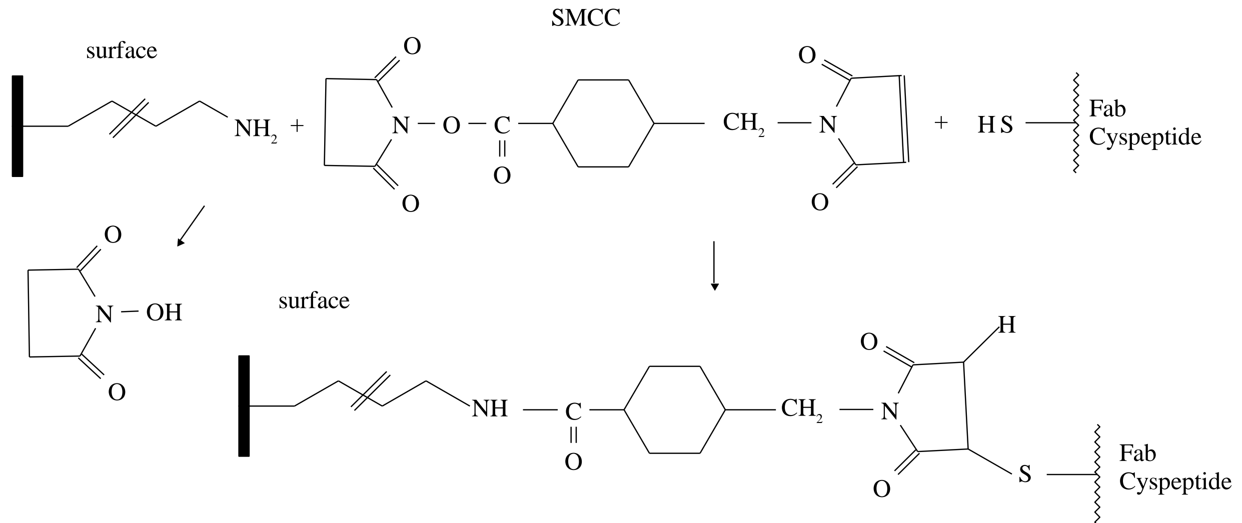
Reaction A



B. Sulfosuccinimidyl maleimidomethyl cyclohexane carboxylate (SMCC).

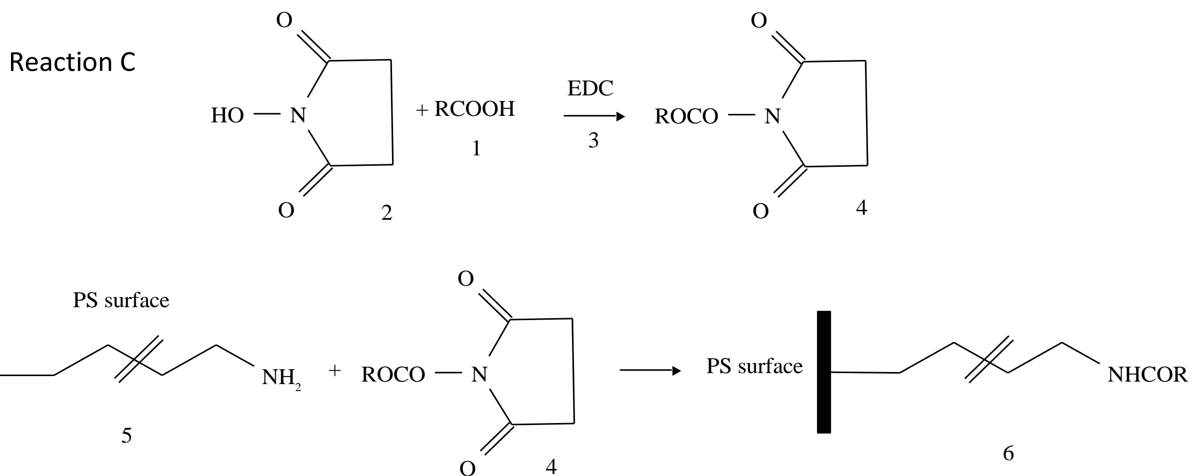
This heterobifunctional linker is capable of linking compounds with SH-containing compounds. It can be used especially for covalent immobilisation of Fab-SH-antibody fragments or terminally cysteinized antigenic peptides, thereby exposing the active ends of these compounds to the liquid phase.

Reaction B



C. N-Hydroxysulfosuccinimide (Sulfo-NHS) or N-Hydroxysuccinimide (NHS) combined with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide Hydrochloride(EDC).

The EDC linker combined with sulfo-NHS is capable of linking small peptides (M.W. around 1000) via their carboxyl group to the NH-activated strips surfaces.



1. Peptide
2. Sulfo NHS (or NHS)
3. EDC
4. Intermediate active compound resulting from the reaction
5. Biomat NH_2 surface
6. Peptide covalently immobilised on Biomat NH_2 surface

TECHNICAL NOTE N. 12

General directions for the use of surfaces with amino and carboxylic groups

Biomat has developed modified polystyrene surfaces introducing chemical groups such as NH_2 and COOH.

These groups are able to covalently bind compounds to the plastic surface. The optical properties of polystyrene remain unchanged, allowing to use the modified surfaces as powerful tools for diagnostic assays.

These surfaces offer the possibility to

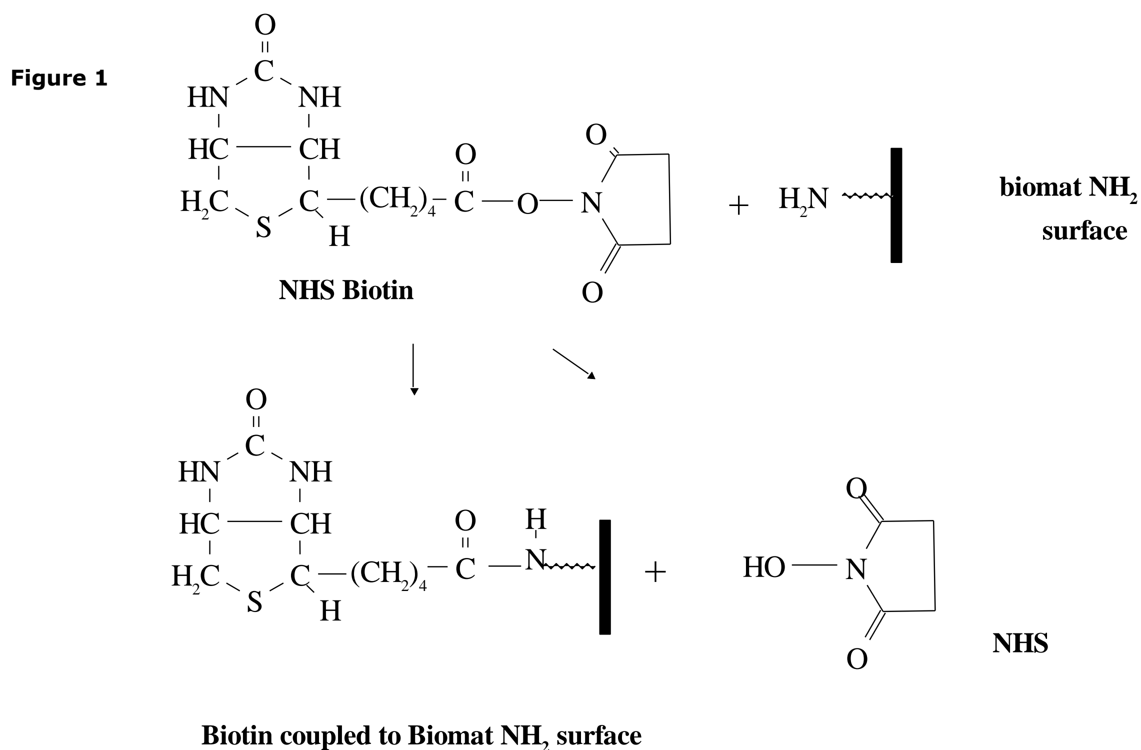
- covalently immobilize small molecules which only bind weakly or not at all by physical adsorption
- orientate the immobilization of molecules in a defined way on the solid phase.

Hereunder are some examples of application that can be used as guidelines to enable users to develop their own bio-specific assays.

1. Coupling of NHS-activated compounds

A very simple and easy application of Biomat NH_2 surfaces is coupling of molecules that have been activated by esterification with N-hydroxysuccinimide derivatives (NHS). In our experiment an-Hydroxysuccinimide active ester of biotin links immediately via its carbonilic group to the surface amino groups as shown in figure 1.

Figure 1



Preparation of reagents and buffers

Materials

Solid phase:	Biomat plates	MT02F2-AM1 (primary amino groups) MG01F-HB (high binding capacity)
ε-Caproylamido-biotin-N-hydroxysuccinimide ester (NHS- biotin)	BIO-SPA	Cat No. B002-61
Dimetilformamide (DMFO)	Fluka	Cat No. 40250
Tween® 20	Merck	Cat No. 822184
Streptavidin	BIO-SPA	Cat. No. S002-60
Streptavidin-peroxidase conjugate	BIO-SPA	Cat. No. SB01-61
BSA	Intergen	Cat. No. 3100
TMB peroxidase substrate	Kirkegard & Perry	Cat. No. 50-76-05

NHS-Biotin stock solution

NHS-biotin	6mg
DMFO	2 ml

NHS-Biotin solution 150μg/ml

NHS Biotin stock solution	500μl
PBS 0.1M pH 7.2+0.15% Tween® 20	→10ml

NHS-Biotin solution 100μg/ml

NHS Biotin stock solution	333μl
PBS 0.1M pH 7.2+0.15% Tween® 20	→10ml

NHS-Biotin solution 50μg/ml

NHS Biotin stock solution	167μl
PBS 0.1M pH 7.2+0.15% Tween® 20	→10ml

NHS-Biotin solution 10μg/ml

NHS Biotin stock solution	33μl
PBS 0.1M pH 7.2+0.15% Tween® 20	→10ml

Streptavidin-mix

Streptavidin	50μg
Streptavidin-peroxidase	1μg
PBS-BSA 1%	10ml

Experiment

1. Add 100μl NHS-biotin solutions 150-100-50-10 μg/ml and 0.1M PBS + Tween® 20 0.15% pH 7.2 as 0 μg/ml to the wells (with primary amines and HB). Seal the wells with adhesive tape to prevent evaporation
2. Incubate overnight at room temperature
3. Empty the wells and wash with 0.1M PBS + Tween® 20 0.05%, pH 7.2 four times
4. Add 100μl of streptavidin mix to each well and incubate 30 minutes at room temperature
5. Empty the wells and wash with 0.1M PBS + Tween® 20 0.05%, pH 7.2 four times
6. Add 100 μl /well of TMB substrate solution and incubate 10 minutes at room temperature
7. Stop the substrate reaction by adding 100 μl of sulphuric acid 1 N and read the optical density values at 450 nm

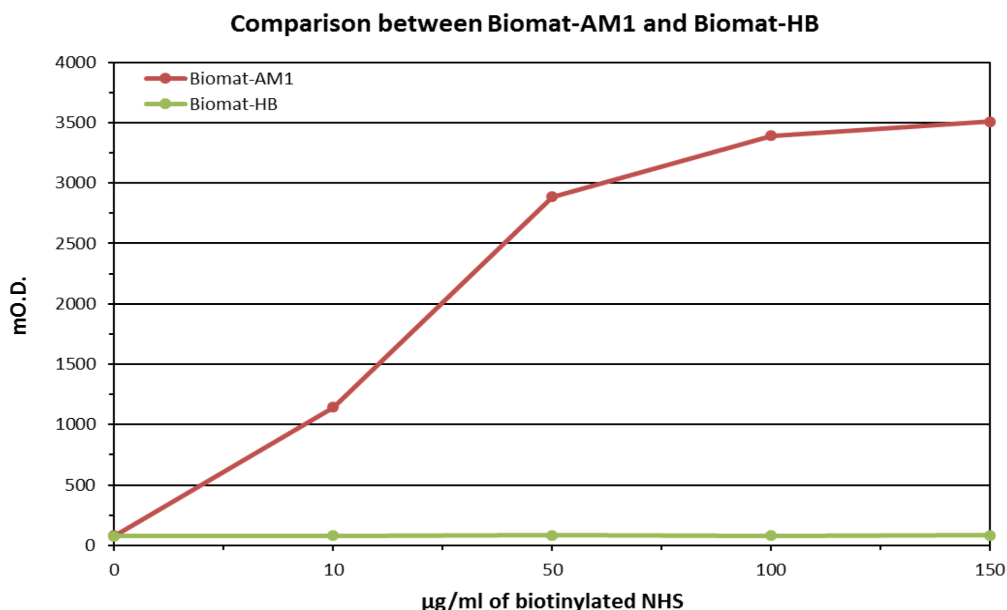
Results

The results (see fig. 2) show a clear correlation between the concentration of NHS-biotin added to the wells and the amount of biotin bound to the Biomat NH_2 surface.

On the other side no biotin is bound onto the plate without primary amino groups grafted to its surface, showing that passive adsorption of neither biotin nor enzyme conjugate occurs.

We therefore conclude that NHS-Biotin has indeed been covalently bound to the amino groups present on the Biomat NH_2 surface.

Figure 2

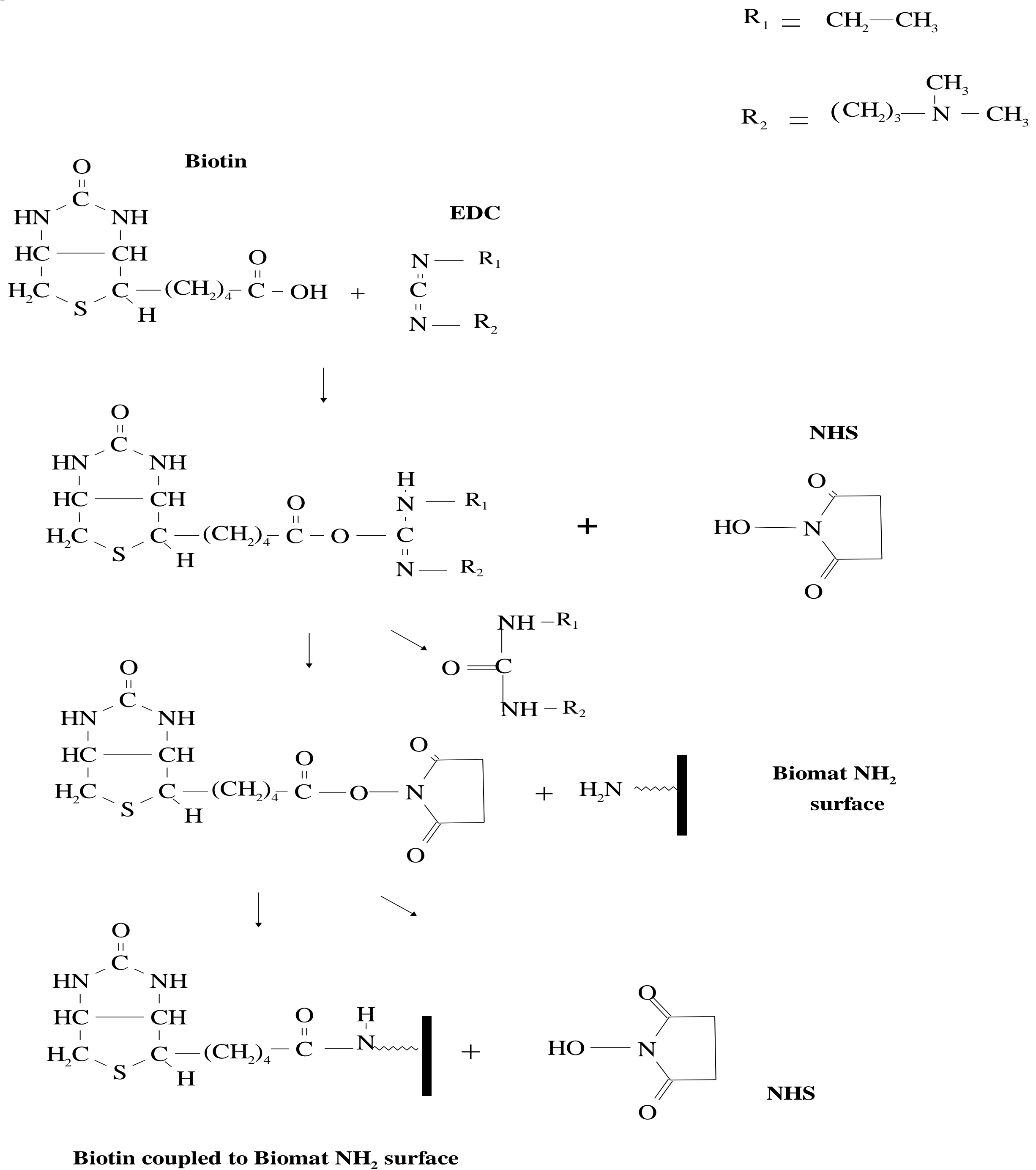


2. Coupling hapten or peptide, having a carboxylic group, to Biomat NH_2 surface

The carboxylic group presents in a molecule with a low molecular weight, such as a hapten or a peptide, binds to Biomat NH_2 through formation of amide bonds between the carboxylic group presents in the molecule and the surface amino group by the combined action of carbodiimide and N-hydroxysuccinimide.

The figure 3 shows the reaction scheme for coupling of the hapten, biotin, through its available carboxylic group.

Figure 3



Preparation of reagents and buffers

Materials

Solid phase:	Biomat plates	MG02F-AM1(primary amino groups) MG01F-HB (high binding capacity)
d-Biotin	Sigma	Cat. No. B 4501
1-Ethyl-3-(3 dimethylaminopropyl)-carbodiimide (EDC)	Sigma	Cat. No. E 1769
Sulfo-N-hydroxysuccinimide (sulfo-NHS)	Fluka	Cat. No. 56485
Dimethylsulfoxide (DMSO)	Merck	Cat. No. 2931
Tween® 20	Merck	Cat. No. 822184
Streptavidin	BIO-SPA	Cat. No. S002-60
Streptavidin-peroxidase conjugate	BIO-SPA	Cat. No. SB01-61
BSA	Intergen	Cat. No. 3100
TMB peroxidase substrate	Kirkegard & Perry	Cat. No. 50-76-05

Biotin stock solution

d-Biotin	7.8 mg
DMSO	0.5 ml
Distilled water	0.5 ml

EDC solution

EDC	5.8 mg
Distilled water	to 10 ml

Biotin/NHS solution

Biotin stock solution	500µl
Sulfo-NHS	3.45 mg
Distilled water+0,30% Tween® 20	to 10 ml

Streptavidin-mix

Streptavidin	50 µg
Streptavidin-peroxidase	1µg
PBS-BSA 1%	10ml

Experiment

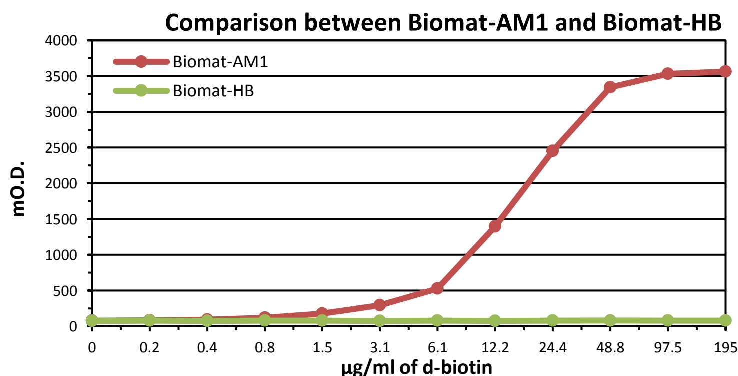
1. Add 50 µl of distilled water to each well, apart from wells in column 2. Then add 100 µl of Biotin-NHS solution to all wells in column 2
2. Dilute by transferring 50 µl from the wells in column 2 to column 3, mix, transfer 50 µl from column 3 to column 4, mix and proceed in this way up to column 12
3. To start reaction: add 50 µl of EDC solution to each column. In blank experiment (column 1) add 50 µl of distilled water instead of EDC
4. Incubate at room temperature for 2 hours
5. Empty the wells and wash with 0.1M PBS+0.05% Tween® 20 pH 7.2 four times
6. Add 100µl of streptavidin mix to each well and incubate for 30 minutes at room temperature
7. Empty the wells and wash with 0.1M PBS+0.05% Tween® 20 pH 7.2 four times
8. Add 100 µl of TMB substrate solution to each well and incubate for 10 minutes at room temperature
9. Stop the substrate reaction by adding 100 µl of sulphuric acid 1 N and read the optical density values at 450 nm

Results

The results of this experiment (figure 4) clearly show that the molecule (biotin) is bound in a detectable way to the Biomat NH_2 (cod. AM1) whereas no detection could be obtained on Biomat HB.

The results indicate that a covalent coupling has taken place between the carboxylic group in the biotin and the primary amino group grafted on the Biomat NH_2.The results (data not displayed) point out that without adding carbodiimide the covalent binding of biotin does not occur.

Figure 4



AMINATED SURFACE Examples of application

TECHNICAL NOTE N. 8

1. DNA binding on secondary amine support

Phosphorylation and labeling of the capture probe

DNA used for carbodiimide-mediated binding is phosphorylated at their 5' end with T_4 polynucleotide kinase and an aliquot is radiolabeled with [γ-^{32}P]-ATP by chromatography on Sephacryl 200 spin column (1 ml) (Pharmacia, Uppsala, Sweden). DNA concentration is then measured by fluorescence using the Hoechst H33258 compound (Labarca and Paigen,1980).

The purity of the fragment can be checked by agarose gel electrophoresis. Radiolabeled DNA is mixed with cold DNA in the ratio of 1:10 in order to reduce the level of radioactivity in the experiments.

DNA binding

Covalent linking of DNA can be obtained by fixation of its 5' end phosphate on the activated amino groups of the plastic (Zamatteo *et al*,1996) (Figure 1). Phosphorylated capture probes for Human Cytomegalovirus detection are denatured for 10 min at 100°C, cooled on ice (10 min), and diluted in ice-cold water (1.54 ng/μl). Ice cold 0.1 M 1-methylimidazole, pH 7.5, is added to obtain a final 1-methylimidazole concentration of 10 mM. The denatured DNA solution is dispensed into the microwells (75 μl/well, 0.7 pmol/well) standing on ice. A fresh solution of 0.04 M 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC) in 10 mM 1-methylimidazole is added to each well (25 μl/well and they are incubated for 5 h at 50°C.

After incubation, the wells are washed three times with washing buffer (0.4 N NaOH, 0.25% Tween 20 at 50°C) 200 μ l/well, then incubated 5 min with washing buffer, and finally washed three times again. Microwells are stored dried at 4°C. After the binding, the wells are cut and the amounts of ^{32}P-labelled DNA bound to the wells were measured by liquid scintillation counting.

2. Peptide Binding on primary amine support

Peptides can be chemically grafted on primary amino group via the free thiol group of a cysteine incorporated in the sequence using heterobinctional reagents such as succinimidyl 4-(N-maleimidomethyl) cycloexane-1-carboxilate (SMCC) (Yashida *et al*,1979: Hashida *et al*,1984) (figure 2) that can be purchased from Pierce Chemical Company (Rokford, USA). This crosslinker consists of an NHS ester and a maleimide group connected with a spacer arm. NHS esters react with primary amine, maleimides react with sulfhydryls.

Grafting can be evaluated by radiochemical assay if a tyrosine incorporated in the sequence is iodinated by oxidizing agent such as chloramine-T (Greenwood *et al*,1963).

Aminated microwells are incubated in a 6.5 10^{-2} mM solution of SMCC in 0.1M phosphate buffer (NHS esters react with primary amines at pH 7-9) during 1 h at room temperature. After 3 washes with phosphate buffer and 3 washes with water, the grafting is achieved by incubating cysteine -containing peptides (6.5 μM) overnight at room temperature, in 0.1 M phosphate buffer (maleimides react with SH groups at pH 6.5-7.5). After 3 washes in phosphate buffer, the wells are cut and the amount of coupled ^{125}I peptide is measured by a gamma irradiation counter.

Other bifunctional reagents such as N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Carlsson *et al*, 1978) can also be used.

References

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Greenwood F.C., Hunter W.M., Glover J.S. (1963) *Biochem. J.* **89**, 114-123.
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