

Colorimetric Determination of Amino Groups of Thermo Scientific Nunc CovaLink NH MicroWell Plates

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Introduction

During the last few years the use of covalent attachment of various molecules onto solid matrices carrying functional groups has been significantly expanded^{1,2,3}. The quantitation of reactive solid supported functional groups is considered very important for optimizing the coupling conditions of ligands. Here we report a simple colorimetric method for the determination of the reactive secondary amino groups, present on the inner surface of Thermo Scientific Nunc CovaLink NH MicroWell plates, using readily available reagents.

According to the method (Fig. 1), the solid supported amino groups react with sulfo-succinimidyl-3-(4-hydroxyphenyl)propionate (sulfo-SHPP), which introduces hydroxyphenyl groups on the solid surface retaining the ability to reduce Cu^{2+} to Cu^+ in alkaline medium⁴. Thus, subsequent incubation of the sulfo-SHPP-treated NuncTM MicroWellTM plates with BCA protein assay reagent, containing bicinchoninic acid (BCA) and CuSO_4 , results in reduction of Cu^{2+} to Cu^+ , the latter forming a chelate complex with the BCA absorbing with a maximum at 562 nm⁵. Finally, the quantitation of the hydroxyphenyl groups introduced on the solid surface, and consequently of amino groups reacted, is carried out using a standard curve of tyramine.

Methods and Results

Sulfo-SHPP stock solution. Thermo Scientific Pierce 100 mg sulfo-

SHPP was dissolved in 1 mL of anhydrous dimethylsulfoxide, and then an appropriate volume of 0.1 M bicarbonate buffer, pH 8.5, containing 3% Triton X-100 (Sigma) was used to prepare a 20 mm solution. Lower concentrations of the reagent were prepared by diluting the stock solution with 0.1 M bicarbonate buffer, pH 8.5.

Washing solution

0.25 M carbonate buffer, pH 11.25.

BCA working solution

Reagent A: Aqueous solution of 1% Na_2BCA (Pierce), 2% $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$, 0.16% $\text{Na}_2\text{tartrate}$, 0.4% NaOH and 0.95% NaHCO_3 at pH 11.25. Reagent B: 4% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. The BCA working solution was prepared just before use by mixing 50 parts of Reagent A with 1 part of Reagent B.

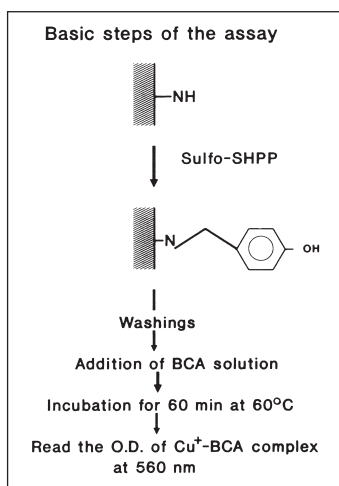


Fig. 1

Basic steps of the assay for the determination of the amino groups present in CovaLink NH MicroWell plates.

Tyramine standard solutions

0.025, 0.05, 0.1 and 0.2 mm tyramine hydrochloride in 0.25 M carbonate buffer, pH 11.25.

Solid Matrices

CovaLinkTM Modules NH F8 (Cat. No. 478042) and Thermo Scientific Nunc Immuno Modules MaxiSorp F8 (Cat. No. 468667) were used in all cases.

Protocol

Add 100 μL of sulfo-SHPP solutions to the wells and incubate for 8 hr. Wash the wells 5 times with the washing solution. Add 10 μL of 0.25 M carbonate buffer, pH 11.25, to the test wells or 10 μL of tyramine standard solutions to control wells (these were treated exactly as the test wells, but the sulfo-SHPP was eliminated from the incubation solution). Add 100 μL of BCA working solution to each well and incubate under mild shaking at 60°C for 60 min. Read the O.D. of the wells at 560 nm subtracting the blank value of the BCA solution. Calculate the amino group content of the wells using a tyramine standard curve (Fig. 2).

Following the described protocol we found that a 2 mm concentration of sulfo-SHPP solution was adequate for maximum measurements of the CovaLink NH wells, whereas the value obtained for the MaxiSorpTM wells (not possessing amino groups) was negligible using concentrations up to 4 mm of Sulfo-SHPP (Fig. 3). As shown in Fig. 4, using a 2 mm sulfo-SHPP solution, maximum values were obtained after an 8 hr incubation period.

Using the O.D. values obtained under the described conditions, we estimated that the amino group content of CovaLink NH MicroWell plates ranged between 0.50 and 0.60 nmole/well with a mean of 0.55 nmole/well.

Discussion

- Here we present a rapid and convenient colorimetric method, which specifically quantifies the reactive amino groups of CovaLink NH MicroWells. The method requires two commercially available reagents; (a) sulfo-SHPP, which reacts with the amino groups and introduces hydroxyphenyl groups on the solid surface, and (b) BCA protein assay reagent, for the titration of hydroxyphenyl groups through their ability to reduce the Cu^{2+} to Cu^+ in alkaline medium. Since other groups (e.g. thiols) have similar reducing properties with the hydroxyphenyl group, several acylating reagents with appropriate physicochemical characteristics may also be used for covalent coupling with the amino groups instead of sulfo-SHPP.
- For this study, we used Triton X-100 in the diluent of sulfo-SHPP in order to increase its solubility and decrease the non-specific binding on the solid surface when high concentrations (>1 mM) of the reagent were used (Figs. 3 and 4). Nevertheless, when concentrations

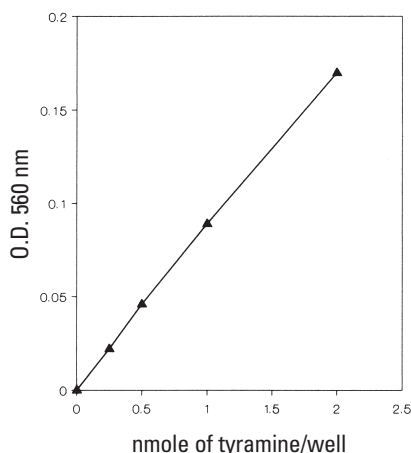


Fig. 2 Standard curve resulting from the reaction of varying tyramine concentrations with BCA working solution, in CovaLink NH MicroWell plates.

of sulfo-SHPP lower than 1 mM are required, Triton X-100 may be eliminated from the diluent. In this case the incubation time should not be more than 2 hr in order to avoid false positive results.

- Careful and uniform washing is of great importance for optimum measurements. Using sulfo-SHPP as acylating reagent, we were able to eliminate the non-specific binding by washing the wells with carbonate buffer, pH 11.25. However, when different acylating reagents are used, appropriate washing protocols should be selected.
- The amino group content of 0.55 nmole/well (approximately 3.5×10^{14} NH groups per cm^2) estimated for the CovaLink NH MicroWell plates is well correlated

with the value of 10^{14} NH groups per cm^2 estimated using radioactive material for the grafting 1. Thus, it seems that most (if not all) amino groups present on the CovaLink NH surface are reactive and accessible to the reagents used.

- The proposed protocol can also be used, with minor modifications, for the determination of amino group content of several solid supports, e.g. plastic beads, tubes or microparticulated solid matrices 6.

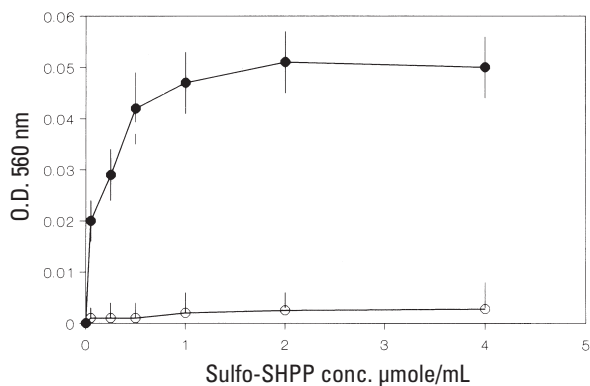


Fig. 3 Plot of O.D. values (mean value \pm S.D., $n=16$), obtained after incubation with BCA protein assay reagent, of CovaLink NH (●) and MaxiSorp (○) MicroWell plates reacted with increasing concentrations of sulfo-SHPP for 24 hr.

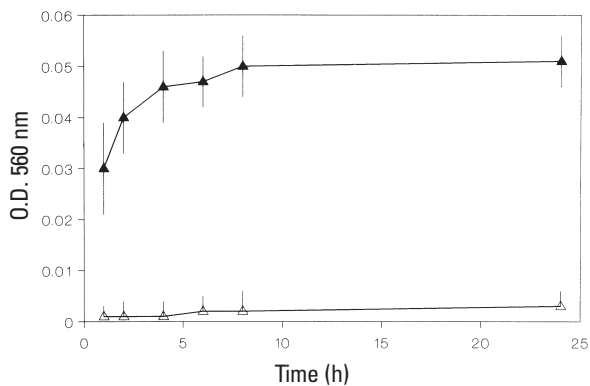


Fig. 4 Time study of the reaction of sulfo-SHPP with CovaLink NH (▲) and MaxiSorp (△) MicroWell plates.

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