Spherotech, Inc.
27845 Irma Lee Circle, Lake Forest, Illinois 60045

SPHERO™ Technical Note STN-16 Rev A 070915

COVALENT COUPLING OF PROTEINS TO MICROBEADS USING A HETEROBIFUNTIONAL COUPLING AGENT

Summary
Covalent coupling of lysozyme to amino-polystyrene particles utilizing a heterobifunctional coupling agent, SPDP, and to aldehyde particles was studied using Particle Concentration Fluorescence Immunoassay (PCFIA). The results showed that these methods can be used to couple lysozyme covalently to amino-polystyrene and aldehyde particles.

The modification of amino-polystyrene particles with SPDP offers the advantage that the resulting pyridyldisulfide particles can be used to react specifically with the sulfhydryl group of proteins, or they can be reduced further with DTT to form sulfhydryl particles. The aldehyde particles can be used to couple covalently to proteins without any coupling agent.

The activity of the lysozyme coated particles depends upon the orientation of lysozyme on the particles. Higher activity was obtained when the amino groups of the lysozyme were utilized to couple to the particles covalently.

Introduction
The coating of proteins to polystyrene latex particles can be accomplished by either physical adsorption or covalent coupling. In general, immunoglobulins can easily be immobilized on plain polystyrene by physical adsorption as described in Spherotech Technical Note # 1, while other proteins may require covalent coupling using a water soluble coupling agent, 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide (EDC). It is often difficult to distinguish between physically adsorbed and covalently bound proteins. Therefore, immunoglobulins or other proteins which can be adsorbed readily onto plain polystyrene particles and also give a quality assay should not be used as a model system to study various means of covalent coupling.

The purpose of this Research Report is to investigate other means of covalent coupling using lysozyme as a model system. Two different covalent coupling methods are chosen for this study. The first one is the use of a heterobifunctional coupling agent, N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP). The second one is the use of aldehyde particles to covalently bind protein without the use of any coupling agent.

Materials and Methods
Dulbecco's Modified Eagle Medium (DMEM) was obtained from GIBCO and supplemented with 10% fetal bovine serum (FBS), 1% normal goat serum (NGS) and 0.1% sodium azide.

Culture supernatants from Hybridoma 5A5, a mouse-mouse line producing IgM antibody specific for lysozyme (Pandex, Mundelein, IL), were diluted with DMEM growth medium to concentrations of 1:10, 1:100, and 1:1000.

Goat anti-mouse IgM-FITC conjugate (Pandex, Mundelein, IL) was diluted to 5 ug/mL with isotonic buffered saline containing 1% FBS and 1% NGS. EDC, Dithiothreitol (DTT) and Lysozyme from chicken egg white were obtained from Sigma. SPDP was obtained from Thermo Scientific Pierce (Rockford, IL). Assay plates (96-well, non-sterial, Thermo Fisher) and Screen Machine™ (Pandex Laboratories, Mundelein, IL) were used during data collection of the fluorescence immunoassay.

Carboxyl-polystyrene (Spherotech, Cat. No. CP-08-10, 0.88µ) and amino-polystyrene (Spherotech, Cat. No. AP-08-10, 0.98µ) particles were prepared by conventional emulsion polymerization techniques and a proprietary functional group coating procedure. An alkyl spacer arm was used to facilitate the covalent coupling. Polyacrolein and polygluteraldehyde particles were prepared by reported procedures described in Spherotech Technical Note # 1 with modifications to

reduce the background fluorescence of the particles. The particles were washed very extensively to remove any residual inorganic salt or emulsifier and resuspended in deionized water at 5% w/v concentration.

SPDP Coupling

A. Preparation of Sulfhydryl Particles

Amino-polystyrene particles (5 mL, 5% w/v) were centrifuged at 3000xg for 15 minutes and the supernatant was decanted. The packed particles were resuspended in 5 mL of phosphate buffered saline (PBS, 0.1M sodium phosphate, 0.1M sodium chloride, pH 7.3). To this suspension was added a SPDP solution containing 12 mg of SPDP in 1.5 mL of ethanol. The mixture was allowed to react at room temperature for at least 90 minutes with occasional gentle stirring. The suspension was centrifuged at 3000xg for 30 minutes and the supernatant decanted. The particles were washed twice with 5 mL of 20% aqueous ethanol and once with 5 mL of 0.1M, pH 5.0 acetate buffer by centrifugation and resuspension. The resulting 2-pyridyl disulfide particles were resuspended in 5 mL of 0.1M, pH 5.0 acetate buffer and reduced with 40 mg of dithiothreitol. After 30 minutes at room temperature with occasional stirring, the particles were centrifuged. The supernatant was decanted and filtered through a 0.2µm cellulose acetate membrane filter. The sulfhydryl group content of the particles was estimated by measuring the absorbance of the supernatant at 343 nm for the released pyridine-2-thione using a molar extinction coefficient of 8.09×10^3 M^{-1}, CM^{-1}. The sulfhydryl group content of the particles was found to be 10 to 15 µmole per gram of solid, depending upon the quantity of SPDP used.

The sulfhydryl particles were washed twice with deionized water by centrifugation and resuspension as above and finally resuspended in 5 mL of PBS to give a 5% w/v suspension.

B. Modification of Lysozyme with SPDP

To a solution containing 4 mg of lysozyme in 2 mL of phosphate buffer (PB, 0.1M, pH 7.0) was added a solution containing 0.865 mg of SPDP in 0.325 mL of methanol. The mixture was agitated at room temperature for 90 to 120 minutes and dialyzed (mol. wt. cut off 1000) against PB. After three changes of buffer in 24 hours, the product was filtered through a 0.2 µ membrane filter. The filtrate was diluted to 40 mL with PB. The O.D. at 280 nm indicated that it contains about 40 µg/mL of SPDP modified lysozyme.

C. Conjugation of SPDP Modified Lysozyme with Sulfyhdryl Particles

A mixture containing 10 mL of 40 µg/mL SPDP modified lysozyme in PB and 0.5 mL of 5% w/v sulfhydryl particles was gently agitated at room temperature overnight. The particles were centrifuged at 3000xg for 30 minutes, washed twice with 10 mL of IBS and once with 10 mL of PB by centrifugation and resuspension as before. The resulting lysozyme conjugated particles were resuspended in 20 mL of PB to give a 0.25% w/v suspension.

Covalent Coupling of Lysozyme to Aldehyde Particles

To a solution containing 1.25 mg of lysozyme in 4 mL of PB (0.1M, pH 6.2) was added 1 mL of 2.5% w/v polyacrolein particles of polyglutaraldehyde coated polystyrene particles and 3 mg of sodium cyanoborohydride. The mixture was gently agitated overnight at room temperature. Bovine serum albumin (BSA, 3 mL, 0.1% in PB) was then added and the agitation was continued for another 2 hours at room temperature.

The particles were centrifuged at 500xg for 30 minutes, washed twice with IBS, and once with PB (0.1M, pH 6.2) by centrifugation and resuspension as before. The particles were resuspended in 10 mL of PB to give a 0.25% w/v suspension.

EDC Coupling of Lysozyme to Carboxyl or Amino-polystyrene Particles

The procedure described in Spherotech Technical Note # 1 was used to couple lysozyme with either carboxyl or amino-polystyrene particles.

PCFIA

Twenty microliters (20 µL) of 0.25% w/v lysozyme-coated particle suspension were added to each well of the assay plate. This was followed by the addition of 50 µL of mouse anti-lysozome antibody (hybridoma %A% supernatant) at dilution factors 1:10, 1:100, 1:1000 and a growth medium control, and 20 µL) of 5µg/mL FITC-labeled goat anti-mouse IgM. After a 15 minute incubation at ambient temperature, the assay plate was manually vacuumed, washed with 100 µL of isotonic buffered saline and read by the Screen Machine. The results were obtained in arbitary fluorescence units.

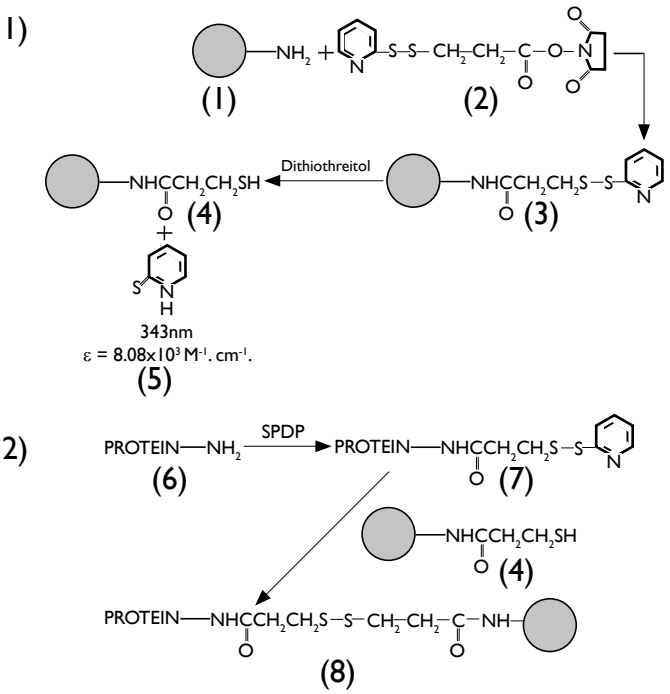
Results and Discussion

As shown in Scheme I, the coupling of protein to amino-polystyrene particles using SPDP involves several steps. The amino-polystyrene particles (1) are first reacted with SPDP (2) to form the pyridyl disulfide particles (3), which are then reduced with dithiotreitol (DTT) to form the sulfhydryl particles (4). If the protein already contains a sulfhydryl group(s), then the pyridyl disulfide particles (3) is used to couple to the sulfhydryl group(s) of the protein. However, if the protein does not contain a sulfhydryl group, then it must be modified first with SPDP to form the SPDP modified conjugate (7), which will react with the sulfhydryl particles (4) to form the protein-particles conjugate (8).

The reaction between amino-polystyrene (1) and SPDP (2) takes about one hour. Longer reaction time of up to four hours does not increase the resulting sulfhydryl content of the particles. However, the sulfhydryl group content of the resulting particles increases with increasing amount of SPDP used.

As shown in Table 1, when 3.38 mg of SPD was used, the resulting sulfhydryl content was 8.87 µmole per gram of solid which accounts for 20.5% of SPDP loaded onto the particles; when 22.4 mg of SPDP was used

SCHEME I



the sulfhydryl content doubled to 17.8 µmole per gram of solid which accounts for only 6.21% of SPDP loaded onto the particles. To save the reagent usage, we normally use 12 mg of SPDP for 5 mL of 5% w/v of particles, which usually gives sulfhydryl content of around 12 µmole per gram of solid. Based on this value, by using the following equations one can calculate the number of sulfhydryl groups per particle and the density of sulfhydryl groups of the surface of the particles.

TABLE 1

SPDP[a] added (mg)	SPDP/solid[b] (µmole/g)	Sulfydryl Content (µmole/g)	% SPDP Loaded
3.38	43.2	8.87	20.5
5.60	71.7	11.50	16.0
11.20	143.4	12.60	8.78
16.8	215.1	16.00	7.43
22.40	286.8	17.80	6.21

a. MW 312.4

b. 5mL of 5% w/v particles were used which is equivalent to 0.25g of solid.

Number of particles: N = $(6W/\pi PD^3) \times 10^{12}$ particles
Surface area of particles: A = $\pi D^2 \times 10^8$ Å^2 / particle
Where : W = Weight of polymer in gram
P = Density of polymer (polystyrene = 1.05)
D = Diameter of particles in micrometer

Since 5 mL of 5% w/v particles contain 0.25 g of polymer, for 0.97 µm particles the total number of particles is 4.98×10^{11}, at 12 µmole per gram the total number of sulfhydryl groups is 1.8×10^{18}, which means every particle contains 3.61×10^{6} sulfhydryl groups. According to equation (2), the surface are of a 0.97 µm particles is 2.96×10^{8} Å^2, hence the density of sulfhydryl groups is 82 Å^2/SH. Due to the steric hindrance, all of the amino groups cannot be converted to sulfhydryl groups. As a result, the actual amino content of the particles would have to be more than the sulfhydryl content listed in Table 1.

SCHEME II

III) —CHO + PROTEIN—NH_2 $\xrightarrow{NaCNBH_3}$ —CH_2NH—PROTEIN
(9) (6) (10)

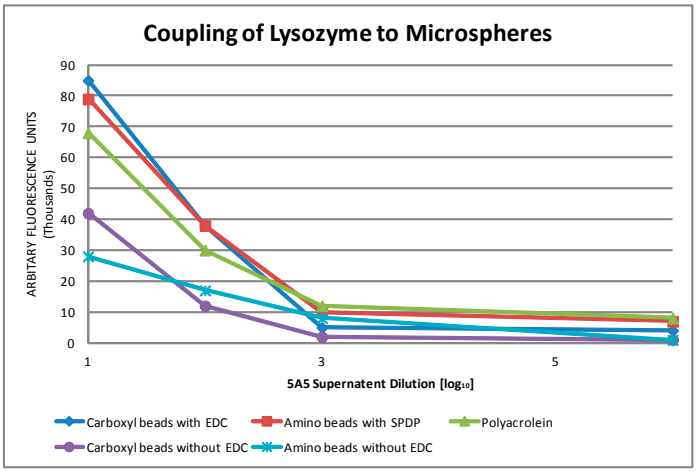
IV) —CHOH + PROTEIN—NH_2 $\xrightarrow{EDC}$ —CONH—PROTEIN
(11) (6) (12)

V) —NH_2 + PROTEIN—COOH $\xrightarrow{EDC}$ —NHCO—PROTEIN
(1) (13) (14)

The coupling of protein to aldehyde particles, carboxyl or amino particles is shown in Scheme II. The use of sodium cyanoborohydride reduces the Schiff's base to more stable secondary amino linkage. Either carboxyl or amino particles can be used to couple lysozyme with EDC. However, the orientation of lysozyme on the resulting particles is not the same. The carboxyl polystyrene would react mainly with the amino group of lysozyme while amino-polystyrene would react mostly with the carboxyl groups of lysozyme, thus exposing different parts of the molecule for antibody binding.

The assay performance of lysozyme-coated particles obtained by various coupling methods is shown in Figure 1. The use of amino-polystyrene particles with and without EDC resulted in a poor assay, but when the amino-polystyrene particles were modified to sulfhydryl particles (4) and coupled to SPDP modified lysozyme (7), good results are obtained. Attempts to couple sulfhydryl particles with unmodified lysozyme resulted in no binding, indicating that there was indeed a covalent linkage between sulfhydryl particles (4) and SPDP modified lysozyme (7) as shown in Structure 8 of Scheme I.

Figure 1.



When carboxyl particles were coupled to lysozyme with EDC the assay performance was improved by about two-fold over coupling without using EDC. The coupling of lysozyme to either polyacrolein particles or polygluteraldehyde-coated particles (not shown) gave good assay performance but with a little higher background fluorescence. The advantage of aldehyde particles is that no coupling agent is required for covalent coupling which in turn avoids the possibility of cross-linking the protein itself.

The orientation of the proteins on the surface of the particles is a very important factor to the activity of coated particles. For lysozyme, it seems that whenever the amino groups of lysozyme are utilized for coupling to the particles the activity of coated particles is higher than when the carboxyl groups of lysozyme are utilized for coupling. A very good example is the coupling of lysozyme to amino particles. When the amino groups of lysozyme are used to react with SPDP (7) and then couple to sulfhydryl particles (4), which are derived from amino particles (1) as shown in Scheme I, the activity is higher than when the carboxyl groups of lysozyme are used to couple to the same amino particles (1) using EDC. Since the same kind of amino particles is used, the amino group's content, reactivity of the amino groups and the steric factors should be the same.

The difference in activity after coating would have to be attributed to the difference in the orientation of lysozyme on the surface of the particles.

Although very little activity is observed for the plain polystyrene beads coated with lysozome, it was found that the plain polystyrene beads actually adsorbed as much lysozyme as carboxyl beads without using EDC (Table 2). Under the same condition they both adsorbed about 60% of added lysozyme. For carboxyl beads the ionic interaction between the carboxyl groups on the beads and the amino groups on the lysozyme is enough to orient the lysozyme so that therre is some activity. But for plain polystyrene the random orientation of lysozyme on the surface of the particles resulted in negligible activity.

TABLE 2
Adsorption of Lysozyme[a] to Plain Polystyrene and Carboxyl Polystyrene Beads

Particles[b]	Starting O.D.	Supernatant O.D.	% Lysozyme Adsorbed
Carboxyl	0.23	0.088	62
Plain	0.23	0.093	60

a. Concentration of lysozyme is 60 µg/ml in 0.1M PBS, pH 7.0 containing 0.5% w/v beads. The O.D. is measured at 280nm.

b. The diameter of the beadsare the same (0.98 µm). Beads are coated the same was as described in the experimental section, except no EDC was added.

In conclusion, we have shown that lysozyme can be covalently coupled to amino-polystyrene particles through either EDC coupling on SPDP modification. It can also be covalently coupled to aldehyde particles without the use of a coupling agent. The activity of coated particles varies with the coupling methods used and depends on the orientation of lysozyme on the surface of the microparticles. In order to optimize the activity of immobilized protein on microparticles, one may want to try various coupling strategies including passive adsorption on particles with different surface charges and covalent coupling using different functionalized particles and coupling agents.