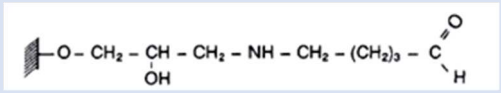
Cellufine™ Formyl

Cellufine Formyl is an aldehyde activated resin for the covalent immobilization of amine containing proteins and ligands. As with all Cellufine products, the base support consists of spherical cellulose beads which exhibit superior rigidity and chemical stability relative to classical agarose gels. Such mechanical strength allows for improved throughput at both the bench scale and on the production floor. The exclusion properties of Cellufine Formyl are similar to those of 4 % agarose gels. Furthermore, due to the robust internal structure and chemical linkage, immobilized ligands show no appreciable leakage. With the use of a condensation agent, ligands (proteins, etc.) can be easily coupled via the reactive aldehyde moiety.

Table 1, Characteristics of Cellufine Formyl

	Characteristics
Ligand	 Formyl (aldehyde)
Matrix	Spherical cellulose particle
Particle size	125 - 210 µm
MW exclusion limit	4,000
Formyl group (µmol/mL-gel)	15-20
Protein immobilization amount (mg/mL-gel)	40 （Depends on protein and reaction conditions）
Spacer length (atom)	8 atom
Operating pressure	＜0.1 MPa
Density	0.7g/ml wet
Storage	0.2M Acetate buffer,pH3.0 containing 0.01 % 2, 2-thio-bis (pyridine-1-oxide)

※Values in Table 1 are not specifications.

Characteristics

- Compatible with high molecular weight ligands and target proteins due to pore size equivalency with 4 % cross-linked agarose media.
- Ligands with primary amino groups can be immobilized.
- Unreacted formyl groups easily converted during reduction to neutral hydroxyls for low non-specific adsorption.
- Built in hydrophilic spacer arms for maximum ligand accessibility and low non-specific adsorption.
- No media damage or fines generation with extended mixing to allow use of simple coupling apparatus.
- Ligand coupling occurs under mild conditions in short reaction times.
- Thermal stability of media allows high temperature reactions.
- Long shelf-life of unreacted media.

Table 2 Immobilized ligands and purification targets for Cellufine Formyl

Immobilized ligand	Target Molecule
Antibodies	Antigens
Antigens	Antibodies
Protein A, G	Antibodies
Lectins	Carbohydrates, Glycoproteins
Cytokines	Receptors
Enzymes	Substrate/Product

Coupling (reductive amination)

The aldehyde active group on Cellufine Formyl reacts with primary amine groups on the ligand to form a Schiff's base complex (see Figure 1). A mild reducing agent is used to convert the Schiff's base to a highly stable linkage. The reducing agent should be selected

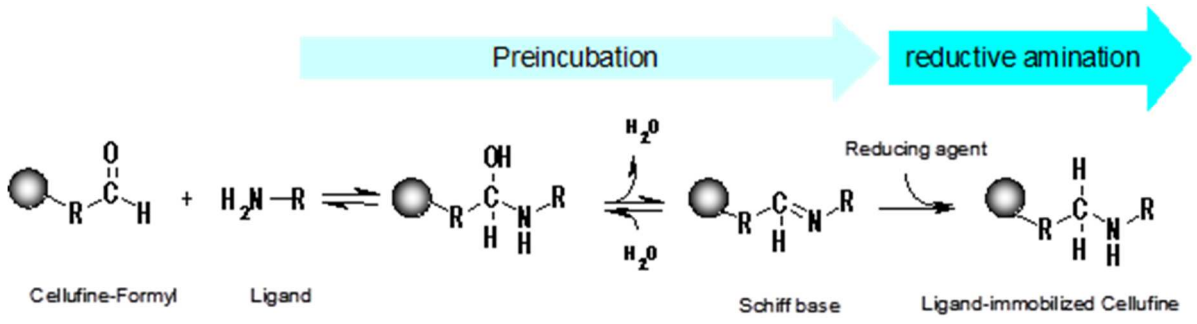


Figure1 Cellufine Formyl Reaction Mechanism

to produce a reasonable reaction rate and yet not be so strong as to damage the protein ligand (such as by reduction of disulfide bonds) or as to reduce the aldehyde groups.

The reaction rate of Cellufine Formyl is rapid enough to be practical, yet slow enough to be extremely gentle to most proteins. It also allows for a fine measure of control. The rate may be controlled effectively with temperature to achieve maximum protein stability. The pH of effective couplings ranges between 3 and 10.

The coupling efficiency (the ratio between amount coupled and amount offered) and total ligand density can be varied and optimized quite easily through changes in coupling ligand concentration, pH and temperature. A standard set of conditions will work well for most cases, but optimization over a broad range can be used to improve process economics for specific applications.

Materials and Methods

Materials

- Cellufine Formyl
- Ligand
- Coupling buffer : The buffer solution which does not contain a primary amine, the range of pH 4 to 11, and concentration are about 0.2M, a phosphate buffere, an acetate buffere, HEPES buffer solution, etc. can use.
- Blocking buffer : 0.2M to 1M of Primary-amine compounds in coupling buffer, such as ethanol-amine, glycine and glycine ethyl ester, etc or 0.2M to 1M of Tirs-HCl buffer pH 7 to 8 is used.
- Reducing agent : the most common reducing agents are sodium cyanoborohydride (SCBH), Trimethylamjneborane(TMAB) and sodium Borohydride (SBH).

Table 3 Types and Characteristics of Reducing Agents

	Advantage	Demerit	Note	pH
SCBH (Sodjum cyano borohydride) $NaCNBH_3$	Protein and aldehyde compound is not reduced, but a Schiff's-base is reduced selectively.	Toxic substance	used at a draft chamber Waste processing necessity	≥ pH4

TMAB (Trimethylamjne borane) $(CH_3)_3NBH_3$	Reduction force is weak and there is almost no damage given to protein.	Solubility is low. (1.3% of solubility to water)	The pre incubation of 1 to 2 hour is required.	≥ pH5
SBH (Sodiumboro hydride) $NaBH_4$	Blocking operation is unnecessary. Toxicity is low.	Since reduction force is strong, a formyl group is reduced. In special protein, use is impossible. (A S-S binding is reduced)	The pre incubation of 1 to 2 hour is required.	≥ pH7

※**Caution:** Solution of SCBH should be made in a well ventilated area, since a small amount of toxic gas will be generated upon solubilization.

Coupling method（Preparation method for 1mL gel）

・Washing

1) Shake the bottle several times to homogenize the slurry in the bottle.（Approximately 50% slurry）
2) Wash 2 mL of the slurry with DI water by decantation or filtration washing until the acetic acid smell is removed. 0.7-0.8 g of gel cake dehydrated by suction filtration for 15 minutes is approximately 1 mL.
3) Add the gel to the reaction vessel and immediately start the coupling operation.

・Coupling

1) Prepare coupling buffer containing ligand.（Ligand concentrations refer to "Coupling Conditions"）Add the ligand solution at a 1 to 2 times of gel volume.
2) Pre-incubation should be reacted for 1 to 2.5 hours. （If using SCBH, pre-incubation is not required) During the reaction, shake or stir with a mild magnetic stirrer. React at a temperature suitable for ligand stability.
3) Add reducing agent (5～10 mg) and continue the reaction for 2-16 hours.(Instead of powder, 0.1 ml of a 50-100 mg/ml reducing agent solution may be added.)
4) A reaction solution is removed by filtration or decantation. washes in several times by coupling buffer 20ml.The amount of coupling can be calculated by measuring the ligand concentration contained in reaction solution and washing solution.

5) 1～2 mL of blocking buffer and 7 mg of reducing agent (same amount as (3)) are added to the washed gel and react for 2 hours. (When SBH is used as reducing agent, blocking operation is not necessary.)
6) A reaction solution is removed by filtration or decantation. Washes in several times by 20ml of coupling buffer 2 or water.
7) The gel is suspended in an appropriate buffer for the ligand and stored at low temperature.

Table.4 The summary of operation (Required quantity per 1mL gel)

1	Cellufine Formyl	preservation solution is washing-removed. 1ml（0.7g to 0.8g- wet-g）
2	Coupling buffer	Coupling buffer solution containing ligand (1 to 2 ml) Refer to the “Coupling Considerations” for ligand concentration
3	Preincubation	agitates gently for 1 to 2.5 hours（preincubation is unnecessary when SCBH is used.）
4	Reducing agent	reducing agent adds 5-10mg.（Instead of powder, it’s may add 50 to 100mg/ml of 1ml reducing agents.）
5	Coupling reaction	agitates gently for 2 - 16 hours It depends for the temperature suitable for a reaction on the stability of a ligand.
6	Washing	A reaction solution is removed by filtration or decantation. washes in several times by coupling buffer 20ml. The amount of coupling can be calculated by measuring the ligand concentration contained in reaction solution and washing solution.
7	Blocking reaction	1-2ml blocking solution is added to the washed gel, and the same quantity as a reducing agent (4) is applied. agitates gently for about 2 hours
8	Washing	A reaction solution is removed by filtration or decantation. Washes in several times by 20ml of coupling buffer 2 or water.

・Affinity chromatography

1) Calculate volume required for the desired bed dimension, keeping in mind that bed compression will occur during column packing. Prepare a 40 – 60 % (v/v) slurry with the appropriate adsorption buffer.

2) Flow adsorption buffer to prepare a gel bed. Set up the bed support, etc. according to the Operating Instructions for the column. Flow adsorption buffer and equilibrate the column.
3) Flow the sample to adsorb the target substance, and then wash the remaining impurities by flowing adsorption buffer.
   ※Adsorption and elution buffers are selected according to the characteristics of the ligand.
4) After washing off impurities, elution buffer flows to elute the target substance.

※Cleaning and Regeneration

The base gel is stable against acids, alkalis, and organic solvents. Washing and regeneration conditions depend on the stability of the ligand. Protein ligands cannot be used under harsh conditions. Insufficient washing and regeneration may cause contamination to accumulate and affect separation during repeated use. Therefore, if the gel is to be used repeatedly for a long period of time, washing and regeneration must be ensured. Wash with both or either acidic or alkaline buffer solutions in which the ligands are stable. The following is a general description of cleaning and regeneration methods.

After washing with 10x volume of 0.5M acetate buffer (pH 3.0), wash with 10x volume of 0.5M phosphate buffer (pH 9.0). Finally, equilibrate the column with 10x volume of equilibration (adsorption) buffer.

Coupling Conditions

Preparation of affinity chromatography resin is affected by ligand concentration, temperature, pH, etc. during coupling. Optimal conditions should be considered. Each condition is shown below.

【ligand density】

See Fig. 1.

①The ligand solution of high concentration(more than 20mg/ml) should be used if;

- High ligand concentration on the gel(more than 15mg/ml gel) is required.
- The substance to be collected on the affinity gel is low in molecular weight and the maximum recovery is attempted.
- The ligand is low in price and available.

② The ligand solution of low concentration (lower than 20mg/ml) should be used if;

・ Low ligand concentration on the gel (lowerthan 15 mg/ml gel) is required in order to avoid steric hindrance of ligands.

・the ligand is high in price and maximum binding to the gel is attempted.

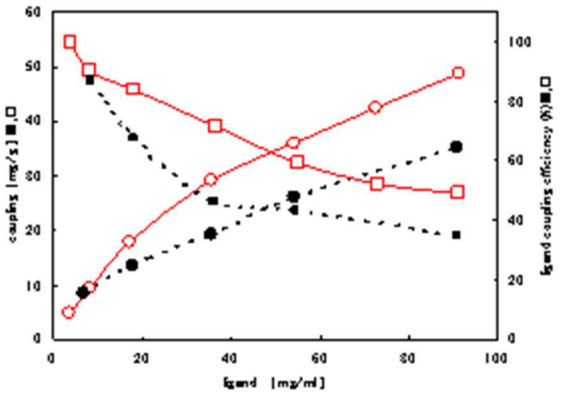


Fig.1 The effect of the concentration of ligand in the coupling reaction.

【pH of coupling buffer】

See Fig.2.

The optimum pH is different for different proteins. The optimum pH should be selected considering the stability of ligand, the binding efficiency.etc. In general, using a pH higher than the pI of the ligand protein can achieve the high binding efficiency.

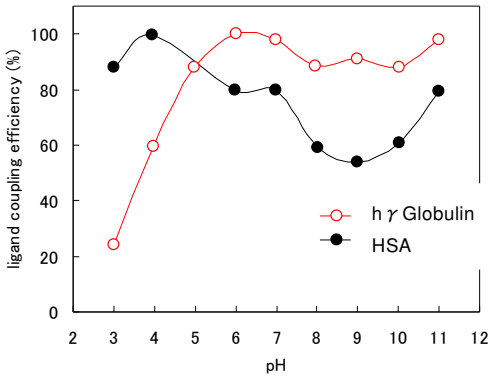


Fig.2 The effect of pH of coupling buffer on the amount of ligand coupled to the gel

【Reaction temperature and incubation time】

See Fig.3.

① The higher the reaction temperature is the more of coupling will occurr, As the general rule applies, the consideration has to be given to the stability of the protein (ligand) at the selected temperature. A suitable temperature for keeping the ligand stable shall be selected.

②In the cases where the ligand is very unstable or the required amount of ligand on the gel is low (less than 10mg/ml gel). the necessary time for incubation is 24 hrs.

③If the maximum amount of ligand is to be coupled to the gel, the stirring of more

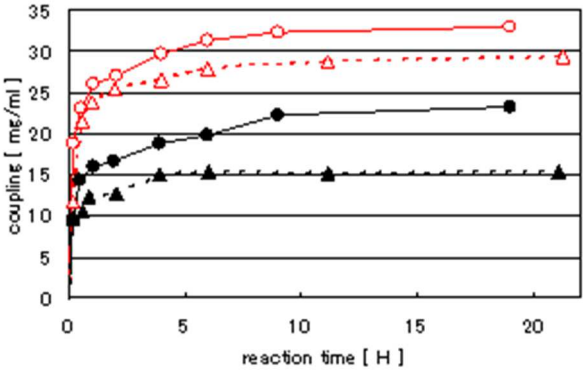


Fig3 The relation between the time for coupling reaction and the quantity of coupled protein.

than 4 hrs. is recommended.

【Pre-incubation time】

In order to couple more ligands, the Schiff base should be formed as much as possible before the addition of the reducing agent. Preincubation time of 1.5 to 2.5 hours is required.

Sodium cyanoborohydroxide (SCBH) liquid waste treatment method

For handling and disposal methods of SCBH, please refer to the MSDS of the manufacturing company or reagent distributor. The following is a list of SCBH disposal methods for reference.

Notes：The operation is performed within a draft.

Method-1

(1) After using SCBH, add the reaction solution and the primary washing solution to a beaker.

(2)Add (1) to a strong alkaline solution of calcium hypochlorite with stirring. At this time, make sure that excess NaOH and calcium hypochlorite remain.

(3)Allow the solution to stand for 24 hours, and then dispose of it as waste.

Method-2

(1)After using SCBH, add the reaction solution and the primary washing solution to a beaker.

(2)Add NaOH solution to make it alkaline.

(3)Add an excess of iron(II) sulfate solution to the reaction solution, let stand for 1 hour, and then waste and dispose of the solution

Sample Preparation and Load

Prepare a 1 – 10 mg/ml solution of sample in adsorption buffer. Remove insoluble material by centrifugation or microfiltration.

Flow rate

20-150 cm/h (＜0.1MPa)

Chemical and Physical Stability

The stability of the coupled gel will be limited by the ligand. However, the base gel is

stable to most salts, detergents, chaotropic agents, 0.1 N NaOH, 0.1 N HCl and can be autoclaved at 121°C for 30 minutes at pH 7.0.

Storage

For storage of opened containers, it is recommended that they be kept in a cold room (2 - 8 °C). Do not freeze. For long-term storage, use 0.2M sodium acetate buffer, pH 3.0, and store at 4°C. Shelf Lifetime is 3 years from manufacture.

Product Ordering Information

	Pack Size	catalog No.
Cellufine Formyl	10 mL	676 944 324
	50 mL	19853
	500 mL	19854
	5 L	19855
	10 L	676 944 335

Purchase/Technical Support

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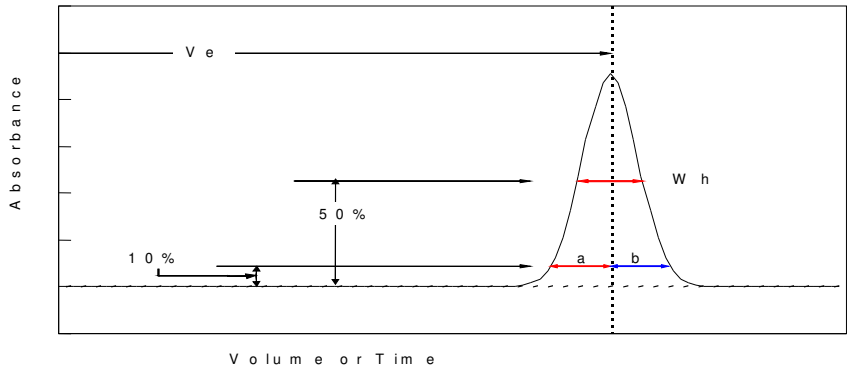
E-mail: cellufine@jnc-corp.co.jp

Appendix 1: Evaluation of column packing

The packing condition of the column is evaluated using indices such as theoretical number of stage plates (N), theoretical stage equivalent height (HETP), and asymmetry (As). These indices are affected by the measurement conditions. For example, these indices vary with column diameter/height differences, piping, solvent sample volume, flow rate, temperature, etc. Therefore, it is necessary to use the same measurement conditions each time. A flow velocity of 30 cm/h is recommended, but higher velocities are possible. However, the higher the flow velocity, the smaller the theoretical number of plates (N). The same conditions (flow rate, column size, mobile phase, and sample) must be used each time to evaluate the column.

Parameter	Condition
Sample volume	1 -2.5% of column volume (CV)
Sample concentration	1-2 %(v/v) acetone (mobile phase：water or adsorption buffer)
	1M NaCl （mobile phase： 0.1-0.4M NaCl aq）
Flow rate (cm/h)	30 cm/h
Detector	UV, Conductivity



L	Column length [cm or m]
V_e	Elution time or volume
W_h	Half of width of peak
a, b	Peak width of 10% peak hight (a) front (b) rear
Note	Ve,Wh and a, b should have same dimensional units

HETP = L/N	$N = 5.54 \times (Ve/Wh)^2$	As = b/a

Generally, number of (theoretical) plates (N) is good if it is over 3000. Acceptable asymmetry factor (As) values range from 0.7-1.5.