CellMosaic, Inc.
10A Roessler Road
Woburn, MA 01801
USA

Phone: 781-463-0002
Fax: 781-998-4694
Email: info@cellmosaic.com
Website: www.cellmosaic.com

SepSphere™ Small Molecule Acid Immobilization Kit (CM71004) User Reference Guide

Contents

CellMosaic, Inc.
10A Roessler Road
Woburn, MA 01801
USA

Phone: 781-463-0002
Fax: 781-998-4694
Email: info@cellmosaic.com
Website: www.cellmosaic.com

Important Notes & Contact Information

READ BEFORE USING ANY RESOURCES PROVIDED HEREIN

The information and methods included in this document are provided for information purposes only. CellMosaic provides no warranty regarding performance or suitability for the purpose described. The performance of this kit during labeling may be affected by various factors, including, but not limited to, the purity and complexity of the starting materials, differences in preparation techniques, operator proficiency, and environmental conditions.

Sample data if provided, is provided solely for illustrative purposes and as examples of a small dataset used to verify kit performance within the CellMosaic laboratory. Information regarding the chemicals and reagents used in the kit is included where necessary.

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c/o CellMosaic, Inc.
10-A Roessler Road, Woburn, MA 01801.
Phone： 781-463-0002
Fax： 781-998-4694
E-mail: info@cellmosaic.com

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Kit Components

This kit provides materials necessary to immobilize a small molecule with a carboxylic acid functional group onto amine-modified agarose beads.

⚠ Upon receipt, remove **Box 1** and store it dry in a refrigerator at -20°C.
Store **Box 2** at 2-8°C.

	Name	Part #	Quantity	Storage condition
Box 1	Reagent A (**green label**)	CM10003	1 unit	-20°C, dry
	Reagent B (**purple label**)	CM10004	1 unit	
Box 2	Amine-modified 4% agarose beads 5 mL	CM71603	5 mL	2-8 °C
	Solution A (**blue label**)	CM01008	1.5 mL	
	Reaction Buffer (**sky blue label**)	CM02006	20 mL	
	Storage Buffer (0.02% NaN_3 in PBS buffer) (**grey label**)	CM02008	60 mL	
	1.5 mL Centrifuge Tubes	CM03CT2	2	
	HPLC Vial with Snap Cap	CM03PT2	2	
	Column Set	CM03SC7	1	
	Centrifuge Tube 15 mL	CM03CT4	1	
	Centrifuge Tube 50 mL	CM03CT5	1	
	Stirrers	CM03PT1	4	
	Airtight Syringe 20 mL	CM03SR1	1	
User Material	Small molecule acid	N/A	NOT PROVIDED (**50 micromole**)	
	Deionized water	N/A	NOT PROVIDED	

Safety Information

Warning: some of the chemicals used in this kit may be hazardous and could cause injury or illness. Please read and fully understand the Safety Data Sheets (SDS) available at CellMosaic.com before storing, handling, or using any of the materials.

Immobilization Chemistry

The kit is designed for use with small molecules containing a single carboxylic acid functional group. The user provides the small molecule. Using the kit components, the carboxylic acid is converted into an activated sulfo *N*-hydroxysuccinimide ester (NHS ester), which subsequently reacts with amine-modified agarose beads.

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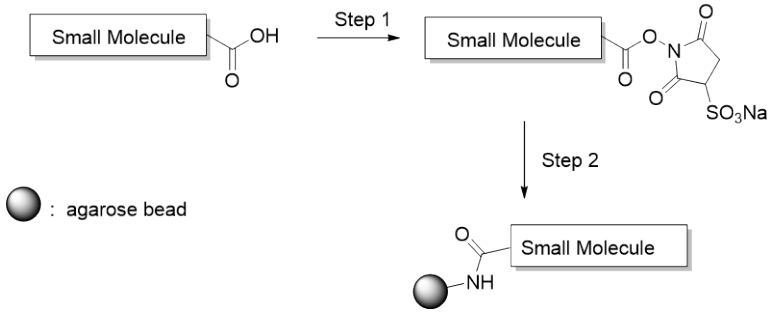

Scheme 1: Activation of small molecule acid and immobilization

Key Features of this SepSphere™ Immobilization Kit:

- **Simplified Immobilization Process**: effortlessly immobilize small molecules with carboxylic acid functional group, requiring minimal setup for immobilization.
- **Comprehensive Kit**: Includes all necessary reagents, buffers, and plasticwares.
- **Efficient and Stable Reactions:** Achieve quantitative immobilization with stable amide bond.
- **Quick and Convenient:** Streamlined preparation with an innovative air-push washing mechanism, requiring only 3 hours total time and less than 30 minutes of hands-on effort.
- **Optimized Agarose Beads:** Utilizes agarose beads with optimized amine functional groups to maximize loading efficiency while preserving the beads' natural properties.

Requirements for small molecule:
1. Preferably > 90% pure
2. Should not contain primary or secondary amine groups
3. Must have a non-hindered aliphatic carboxylic acid
4. For molecules containing aromatic carboxylic acids, hindered aliphatic carboxylic acids, or hydroxyl groups, please consult CellMosaic prior to conducting the experiment.

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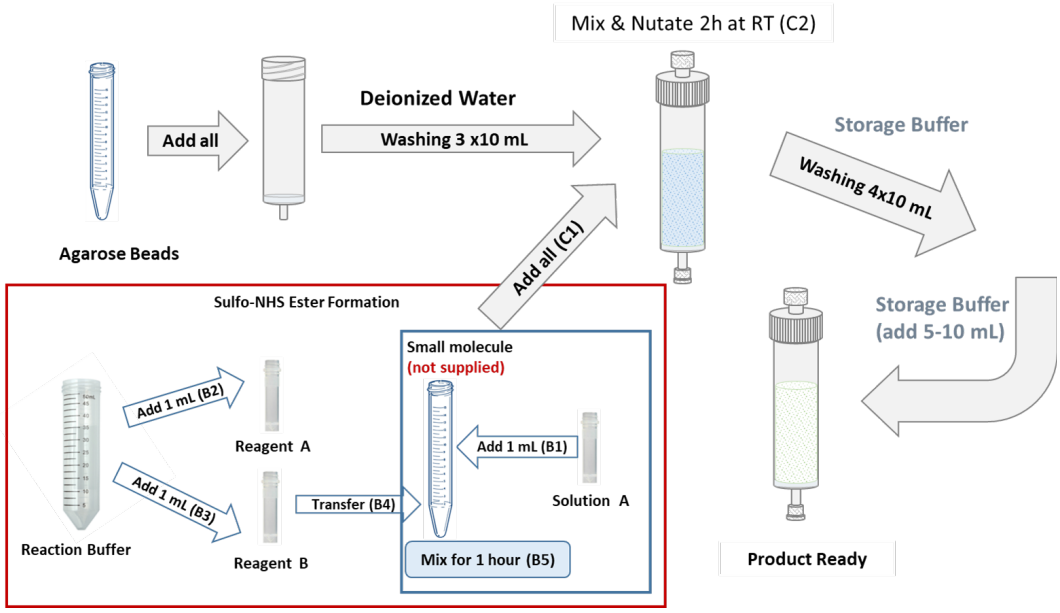
Protocol



Scheme 2. A schematic diagram illustrating the workflow for small molecule acid immobilization, starting with 5 mL of amine-modified agarose beads.

1. Lab Instrumentation Needed

- Vortex mixer
- IEC clinical centrifuge for quick spin (if possible)
- Pipettes and tips
- Timer
- Nutating mixer or 3-D rocker
- Support, clamp, and beaker for waste collection

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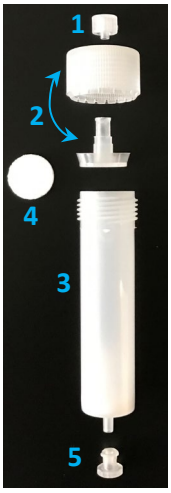
2. Immobilization Lab Techniques at CellMosaic (for reference)

CellMosaic employs a universal workflow and kit design that facilitates the immobilization of small molecules and large biomolecules, the washing of unreacted starting materials, and column packing—all within a single column set-up suitable for any lab environment. The immobilization efficiency achieved in the column is comparable to that of a regular reaction vessel. Washing is conducted using a straightforward manual air-push mechanism with a syringe, eliminating the need for gas/air flow or vacuum systems. The table below outlines the kit components and key techniques employed by CellMosaic for immobilization. Please refer to these techniques while performing immobilization as outlined in the user manual.



Column Design and Setup

Design: The column consists of five components (see left picture):

1. **Male Luer lock cap** – Referred to as the "**top cap"** in the protocol. Used during mixing/nutation.
2. **Column top with Luer attachment** – Referred to as the "**column top"** in the protocol. The Luer attachment ensures a tight seal and allows syringe attachment for washing. The column top is removed for buffer addition and stirring.
3. **Column body** – Contains one polypropylene frit at the bottom and is referred to as the "**column"** in the protocol.
4. **Extra frit** – Provided for column packing after immobilization and referred to as the "**frit"** in the protocol.
5. **Bottom female Luer lock plug** – Referred to as the "**bottom plug"** in the protocol. Used during mixing/nutation and for storage.

Setup: (See right picture) Secure the column to a support stand, lab frame, or any support rod. Position a beaker or a flask beneath the column to collect waste during the process.

Use of Buffer(s) with Resin in Column

Design: All of CellMosaic's buffers are supplied in small, easy-to-use tubes that can be poured directly into the column. Washing buffers are pre-measured to provide the exact volume needed for optimal washing. The total wash volume is fixed, regardless of the volume poured during each wash cycle.

Procedure:

1. Remove the top cap.
2. Unscrew the column top and bottom plug.
3. Place the removed components on a clean surface for reuse later.
4. Pour 8-10 mL of **buffer** into the column containing resin for immobilization (~2 volumes of the resin bed).

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Mixing Resin in Column

Design: The kit includes one or more blue polypropylene stirrers, appropriately sized for the individual columns. These stirrers are resistant to common solvents, and resin generally does not adhere to them.

Procedure for Mixing Resin with Washing Buffer:

1. Using a clean stirrer, mix the resin with the washing buffer for approximately 5-10 seconds. Ensure the stirrer reaches the bottom of the column and the resin is thoroughly mixed.
2. Remove the stirrer, ensuring no resin adheres to it. Set the stirrer aside for reuse during subsequent washing steps.

Procedure for Mixing Resin with Reaction Buffer:

1. Using a clean stirrer, mix the resin with the reaction buffer for 10-30 seconds. Ensure the stirrer reaches the bottom of the column and the resin is thoroughly mixed.
2. Remove the stirrer, ensuring no resin adheres to it. Dispose of the stirrer as solid waste.

Note: For certain reactions, gas or air bubbles may form. Stir slowly and continuously until most of the bubbles are eliminated before capping the column for mixing (nutation).

Washing Resin after Stirring

Design: The kit includes a 20 mL airtight syringe with a slip tip, designed for pushing air through the column during the washing process.

Procedure for Washing:

1. Attach the column top to the Luer attachment after stirring.
2. Draw 20 mL of air into the syringe and securely attach it to the column top.
3. Push the air through the column using the syringe.
4. Once the plunger reaches the bottom, detach the syringe and repeat the air-drawing and purging process.

   Note: After several cycles, approximately 1.2 mL of residual liquid will remain. This is acceptable; you may proceed to the next washing step.
5. Remove the column top, refill the column with buffer, and repeat the washing and purging process as needed.

Procedure to Remove Residual Liquid:

If residual liquid needs to be removed for specific reactions (refer to individual protocol), follow this additional step after air purging with the syringe:

1. Place the column into a 50 mL centrifuge tube with the column top attached, but without the top cap.
2. Prepare a counterbalance by filling another 50 mL centrifuge tube with water.
3. Insert both tubes into a quick-spin centrifuge (e.g., IEC clinical centrifuge).
4. Set the centrifuge to its maximum setting and spin for 15-20 seconds.

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Alternative Method:

You can also use conventional centrifuge equipment and spin at ≤750 x g for 1 minute.

Column Setup for Immobilization:

After adding reagents as outlined in the protocol, attach the column top and securely cap/plug the column top and bottom. Ensure all connections are tight and check for any leaks before placing the column on a nutator or mixer. If a mixer or nutator is unavailable, leave the column open with the stirrer in place. Manually stir the resin every 5-10 minutes for the specified duration.

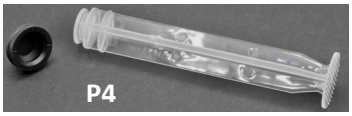
Storage Buffer and Alternatives:

The kit includes a standard storage buffer (1x PBS with 0.02% NaN_3). If you prefer to use your own buffer, substitute it during the final washing step.

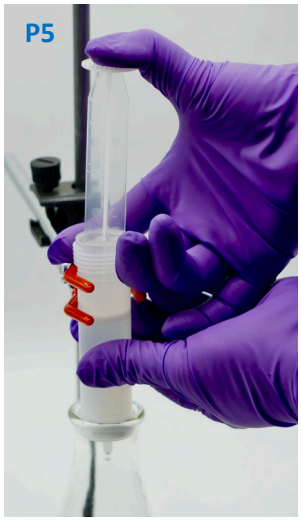
Column Packing:

Design: After the immobilization, the resin can be packed into the column using the included polypropylene frit. Alternatively, you can store the resin in the column and pipette it out as needed for use.

Procedure:



1. Remove the top cap. Unscrew the column top and bottom plug.
2. Fill the column with deionized water or buffer up to the top. Allow a few minutes for most of the resin to settle.



3. Wet the frit with deionized water before placing it inside the column.
4. Remove the black rubber cap from the plunger of the 20 mL syringe.
5. Use the syringe plunger to push the frit into the column, ensuring it goes all the way down to the top of the resin. Apply even pressure to ensure the frit is flat and not tilted. Ensure there is no air trapped between the frit and the resin as you push the frit down.
6. Once the frit reaches the top of the resin, apply slight pressure to secure it tightly in place.
7. Attach the column top with the Luer attachment securely and cap the top.

The column is now ready to use. You can operate it using gravity flow or connect it to a peristatic pump with Luer lock adapters.

The Protocol for immobilization begins on the next page

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3. Preparation of Agarose Beads

Items Needed: Amine-modified agarose beads (CM71603), Column set (CM03SC7), Airtight syringe 20 mL (CM03SR1), Stirrer (CM03PT1), 50 mL centrifuge tube (CM03CT5), Reaction buffer (CM02006, sky blue label), Clean flask for collection (not provided in the kit), Deionized water (not provided in the kit).

A1. **Setup the Column:** Securely attach the Column to a support stand, lab frame, or any support rod and place a beaker or flask under the column for collection. Remove the top cap, unscrew the column top and bottom plug, and set them aside on a clean surface for later use.

A2. Transfer Agarose Beads: Slightly centrifuge the tube containing agarose beads to ensure no bead remain in the cap before opening. Pour all the beads into the Column.

A3. **Wash the Beads**: Pour 8-10 mL of deionized water into the column. Stir the resin with a clean stirrer for 5 seconds and set the stirrer aside for reuse.

A4. Flush Liquid: Attach the column top and use the 20 mL syringe to push the liquid out.

A5. Repeat Washing: Remove the column top and repeat the washing process (**Steps A3 and A4**) **two more times**. Dispose of the stirrer after washing is complete.

A6. Remove Residual Liquid: Place the column into a 50 mL centrifuge tube. Counterbalance with another 50 mL centrifuge tube containing water. Spin the tubes in a quick-spin centrifuge (e.g., IEC clinical centrifuge) at maximum speed for 15 seconds

Note: After spinning, retain the emptied 50 mL centrifuge tube for use in **Step C3** to collect unreacted small molecule acid.

A7. **Add Reaction Buffer:** Securely plug the column bottom with the plug. Add 7 mL of Reaction Buffer (sky blue label).

4. Sulfo-NHS Ester Formation (50 µmol scale)

Items Needed: Reagent A (CM10003, green label), Reagent B (CM10004, purple label), Solution A (CM01008, blue label), 15 mL Centrifuge tube (CM03CT4).

B1. Weigh 50 µmol of the Small Molecule into a 15 mL centrifuge tube. Add 1 mL of Solution A and vortex for 30 seconds or sonicate to ensure complete dissolution. Discard any unused Solution A as hazardous chemical waste.

Calculation:

Amount of the small molecule (mg) = Molecular Weight (MW) of the small molecule x 0.05

B2. Add 1 mL of Reaction Buffer to the Reagent A tube (green label). Vortex for 30 seconds and centrifuge to bring the liquid to the bottom.

B3. Add 1 mL of Reaction Buffer to the Reagent B tube (purple label). Vortex for 30 seconds and centrifuge to bring the liquid to the bottom.

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B4. Transfer the Reagent A solution from **Step B2** to the small molecule solution (**Step B1).** Transfer the Reagent B solution from **Step B3** to the same tube.

B5. Vortex for 30 seconds and centrifuge the tube to bring all liquid to the bottom. Incubate the mixture at room temperature for 1 hour.

5. Immobilization

Items Needed: Agarose Beads from Step A7, NHS Ester Solution from Step B6, Stirrer (CM03PT1), 50 mL centrifuge tube from Step A6, HPLC vials with snap cap (CM03PT2)

C1. Transfer the entire NHS ester content (**Step B6)** into the column containing agarose beads (**Step A7).** Securely cap the column.

Optional HPLC Sample (Sample C1). Mix the column for 30 sec with a Stirrer. Transfer 200 µL of liquid to a 1.5 mL centrifuge tube. Centrifuge at 14,000 x g for 1 minute and transfer 100 µL of the supernatant to an HPLC vial labeled **Sample C1**.

C2. Nutate the column at room temperature for 2 hours.

C3. Attach the column back to the lab stand. Place a 50 mL centrifuge tube under the column to collect unreacted small molecule solutions. Open the bottom plug and push the liquid out with the syringe. Wash the end plug with deionized water for reuse.

Optional HPLC Sample (Sample C3). Transfer 100 µL of the unreacted solution to an HPLC vial labeled **Sample C3**.

6. Washing and Storage

Items Needed: Airtight Syringe 20 mL (CM03SR1), Stirrer (CM03PT1), Storage Buffer (CM02008, grey label).

D1. Remove the column top and pour in 8-10 mL of Storage Buffer. Stir the resin for 10-30 seconds with a clean stirrer. Replace the column top and push the liquid out using the syringe.

D2. Repeat this process (**Step D1**) three times, disposing of the stirrer after use.

D3. Securely plug the column bottom and add 5-10 mL of Storage Buffer. Reattach and securely cap the column top. Store the column at 2-8°C in a refrigerator. *Do not freeze.*

The Agarose Beads are Ready for Your Experiment

The immobilization reaction is typically completed within 2 hours, and analysis is optional. The approximate small molecule loading is 10 µmol per mL of settled beads if fully loaded.

Specification for your product

Matrix: 4% beaded agarose (source: GE Life Sciences, Sepharose® 4B)

Particle Size: 45 – 165 µm

Ligand Concentration: ≤10 µmole per mL of settled beads

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Other Considerations

1. Determine the Loading Capacity by HPLC

The amount of small molecules loaded onto the resin can be determined based on the quantity of the small molecules consumed during the reaction. If you are familiar with HPLC, you can analyze two samples yourself. Alternatively, you may send the samples to CellMosaic for analysis.

HPLC Conditions

Buffer A: 0.1% TFA in water **Buffer B**: 0.1% TFA in acetonitrile
Method: linear gradient of AB solvent (5% B to 95% B over 12 minutes, then held at 95% B for an additional 3 minutes)
Flow rate: Determined by your column (typically 1 mL/min).
Injection amount: 5 µL

Calculation of Loading Capacity

The loading capacity (µmole per mL) is calculated as follows:

$$Loading\ Capacity = \frac{(HPLC\ area\ of\ Sample\ C1 - HPLC\ area\ of\ Sample\ C3) \times 10}{HPLC\ area\ of\ Sample\ C1}$$

Where:
Sample C1 = Pre-reaction sample
Sample C3 = Post-reaction (unreacted) sample

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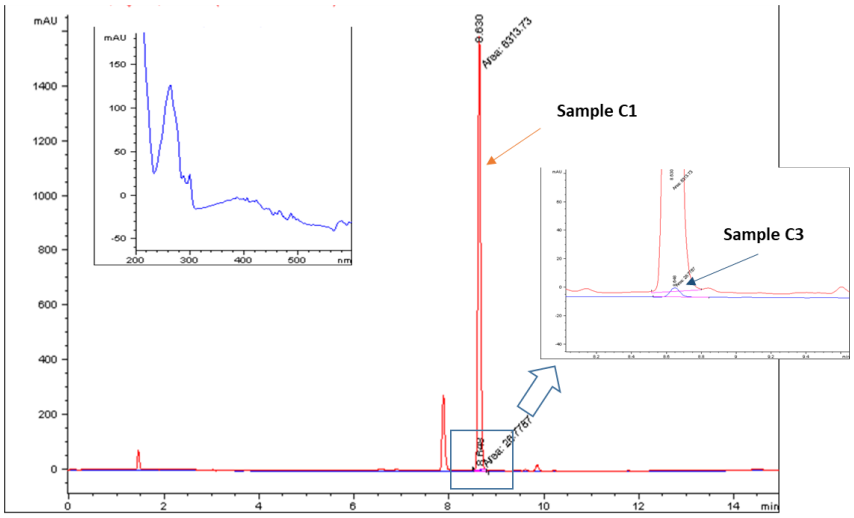
Appendix: Typical Kit Performance Data (LC Analysis, CellMosaic)

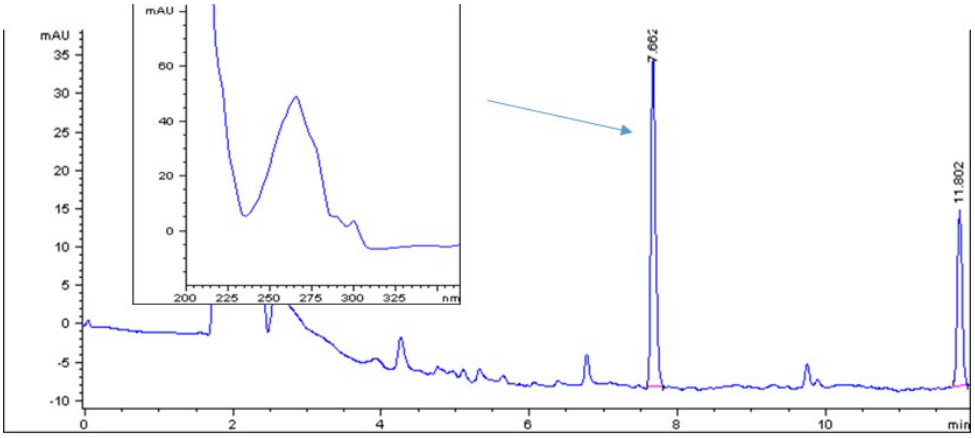
Small molecule information: Fmoc-Gly-OH

Figure 1: C18 HPLC/UV analysis of Fmoc-Gly-OH before (sample **C1**) and after immobilization (sample **C3**) (detected at 254 nm). Left inset: UV spectrum of Fmoc-Gly-OH peak at 8.63 min. Right insert: expanded view of the HPLC profile from 8.0 to 9.6 min.



Calculate Loading: (6313.73-26.7787)x10/6313.73= 9.96 µmole per mL

Figure 2: C18 HPLC/UV analysis of deprotected resin. Inset: UV spectrum of Fmoc-piperidine adduct peak at 7.662 min.



Experiment for analyzing the Fmoc group loaded onto the resin: After immobilization of Fmoc-Gly-OH, approximately 100 µL of beads were removed and added to 200 µL of 20% piperidine in NMP. After mixing for 15 minutes, 5 µL were removed for HPLC analysis. The HPLC profile obtained was identical to the profile from Fmoc-Gly-OH deprotection, confirming that Fmoc-Gly-OH was loaded onto the resin.