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ORIGINAL CONTRIBUTION

# Quantitative evaluations of surface-concentrated amino groups on monolithic-type solid supports prepared by copolymerization method

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**Abstract** We have prepared novel monolithic-type highly cross-linked solid supports having surface amino group using a copolymerization method of functional monomers and cross-linking monomer. From the view point of controlled surface functionalities, we have firstly certified and proved quantitative determination methods of the surface amino groups to utilize and evaluate those solid supports prepared as affinity resins. We utilized a typical titration method and ninhydrin method that have been effectively utilized for quantitative determination of primary amine and amino acid. The ninhydrin method was calibrated using octylamine as a standard compound ($R^2$ = 0.998) and proved applicability for the quantitative determination of other primary amines. A commercially available solid support, Affigel, was able to be quantitatively evaluated only by the ninhydrin method, while Toyopearl afforded just compatible amino group density to the value certified by the manufacturer only through titration method. The obtained incompatible results using both determination methods have been unclear at this moment. On the other hand, both determination methods afforded compatible amino group density of the prepared solid support. The obtained value was up to 126 μmol/ml, which was 94% of the calculated theoretical value based on the feed composition. The results suggested surface-concentrated introduction of amino groups. Based on the determination methods, especially ninhydrin method, we prepared the monolithic-type solid supports having various densities of surface amino groups and proved quantitative and surface-concentrated introduction of amino groups. Those amino groups were able to react with a relatively large ligand molecule, methotrexate, quantitatively and also with controlled density. The obtained results strongly suggest that the novel monolithic-type solid supports have possible advantages when we utilize those as affinity resins for target peptide analyses.

**Keywords** Polymer monolith · Solid support · Surface property · Affinity gel · Co-polymerization

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## Introduction

Solid support materials as well as their tools have been efficiently utilized for organic syntheses as well as peptide syntheses, etc., because those materials are easily removed by filtration or centrifugation from the reaction media, which may make purification process much easier and side reactions may be also minimized. It has been believed that mass transfer inside the solid matrix is highly restricted; therefore, commercially available slightly cross-linked solid supports tend to be generally utilized for those purposes. However, the solid support would take up various topics, so tailor-made solid support will be required for each purpose. Numerous studies have been made to create novel solid supports (http://www.rapp-polymere.com/preise/tent_s_d.htm; [1–10]).

Solid supports, of course, covalently involve reactive functional group(s) utilized for further immobilizing reaction; in addition, those functional groups should be placed on the surface of solid support. This is simply because reactivity of the functional groups must be quantitatively guaranteed. Greater amount of surface functional groups presumably limits the reactivity of those functional groups due to steric hindrance; therefore, a lot of studies have been established.

Usually, those functional groups are introduced onto the "ready-made" solid supports; therefore, the introduced

functional groups must be placed on the solid surface. In this case, the solid base supports were prepared by some polymerization process to produce desired shape, and, after the preparation of solid base supports, appropriate functional groups were covalently immobilized to coat the surface of solid base supports through chemical reaction(s). The immobilized functional groups are directly utilized for further immobilization; however, once the immobilization reaction of functional groups is incomplete, chemically and physically heterogeneous surface will affect the following reactions and experiment quite seriously.

On the contrary, copolymerization method using functional monomers and cross-linking monomers has been traditionally utilized to prepare solid supports. This method is simply easy, and one-step introduction of the functional groups can be done only through the polymerization. A crucial disadvantage of this method is that functional monomers are potentially polymerized to be placed inside the cross-linked polymer matrices. Those "buried" functional groups cannot be effectively consumed for further immobilization reactions [11, 12]. From this point of view, copolymerization method has tended to be excluded for preparation of solid supports.

As mentioned above, the introduced functional groups should be qualitatively and quantitatively controlled on the solid support, if fine control is required for final purposes. First of all, certified quantitative determination methods as well as qualitative evaluation method should be established to evaluate the solid support surfaces. The resulting qualitative and quantitative uniformities of the functional groups on the final solid supports will seriously affect solid-phase-supported experiments, especially affinity experiments.

The functional groups mentioned above are presumably reactive functionalities such as carboxylic acid or amino groups when bioactive and/or unstable compound is immobilized through the functional group because simple condensation reaction can be applied. Therefore, titration method is one of the desired methods for quantitative determination of the functional groups on the solid support [13, 14]. Usually, several times of titration consequently minimize determination error; however, in the case of determination of the solid support, relatively large volume of the solid support should be required to obtain significant titration value. Usually, those solid supports are often rare and valuable; therefore, the titration method is practically inconvenient. In addition, the other acidic and/or basic functionalities on solid support and decomposition of solid support will badly affect the titration results.

In affinity experiments, quantity and quality of the immobilized ligands were reported to affect the experimental results seriously [9, 10, 15–20]. For example, number of effective ligands, in other words, surface density of ligands, is one of the most important requirements to capture target proteins effectively on affinity resins. Optimization of number of effective ligands will be necessary to realize exact and appropriate interactions between a small molecular ligand and the target proteins.

If we discuss about the amount of captured target proteins, the density of ligands on the solid support must be the highest value. However, if the target proteins are sterically bulky and interaction sites are placed deeply inside the proteins, the density of ligands should be decreased. We have to break out of the dilemma. Therefore, microenvironment around the immobilized ligands should be controlled to be optimized for each affinity experiment. This kind of strategy will be very important for future affinity resins. This is because affinity experiment on solid support is nothing more than in vitro experiment.

In this paper, we have prepared novel monolithic-type highly cross-linked affinity resins having a variety of amino group density using copolymerization method based on our previous works [1, 2]. The introduced amino groups were quantitatively evaluated by titration method as well as ninhydrin method [21–23]. Ninhydrin method was well known as a quantitative determination method applicable for amino acids and primary amines; therefore, we carefully checked the applicability of this method to commercially available affinity resins reported to have amino groups as the functional group.

In addition, we theoretically calculated amino group density of the prepared monolithic-type affinity resins and compared those values with the values determined by titration method as well as ninhydrin method. Finally, reactivity of the amino group introduced was evaluated using relatively bulky ligand, methotrexate (MTX), to check qualitative ligand environment using our prepared affinity resin and a commercially available affinity resin.

We do know this kind of work belongs to "old"-fashioned work, but the results shown in this work will be very important for future solid-supported reactions and experiments.

## Experimental section

### Reagents

Solvents and reagents were utilized without further purification unless it was particularly mentioned. Monomers and porogen were structurally illustrated in our previous report [2].

Nacalai Tesque, Inc. (Kyoto, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and Bio-Rad Laboratories (Tokyo, Japan) were abbreviated to be shown simply as Nacalai, Wako, TCI, and Bio-Rad in the following experimental description.

Trifluoroacetic acid and ninhydrin were purchased from Nacalai. 2,2′-Oxydiethanol (DEG-p), 2,2′-azobis(2,4-

dimethylvaleronitrile) (ADVN), oxalic acid, 0.1 N NaOH:1 mol/l sodium hydroxide solution, 1 mol/l hydrochloric acid, 1.0% *w*/*v* phenolphthalein ethanol (90) solution, octylamine, dodecylamine, 3-phenyl-1-propylamine, ethanol (EtOH), pyridine, acetonitrile, and (*S*)-2-(4-(((2,4-diaminopteridin-6-yl)methyl)(methyl)amino)benzamido)pentane-dioic acid (MTX) were purchased from Wako. 2-(2-Methoxyethoxy) ethyl methacrylate (DEG-m) and 2-(3-benzoyl phenyl)-propionic acid (ketoprofen) were purchased from TCI. 4-Carboxybenzenesulfonamide (sulfonamide) was purchased from Sigma-Aldrich (Tokyo, Japan). DEG-m was purchased from TCI. *N*-Methyl-2-pyrrolidinone dehydrated (dry-NMP) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (water-soluble carbodiimide: WSCD) and 1-hydroxybenzotriazole (HOBt) were purchased from Peptide Ins. (Osaka, Japan). *N*-*t*-Butoxy-17-amino-3,6,9,12,15-pentaoxaheptadecane-1-nyl methacrylate (Lig-m) was kindly donated by Reverse Proteomics Research Institute (Tokyo, Japan). Polyethylene glycol #400 dimethacrylate 9G NK ESTER (9G) was donated from Shin-Nakamura Chemical Co., Ltd. (Wakayama, Japan). Toyopearl AF-Amino-650 M (Toyopearl) was purchased from Tosoh Bioscience (PA, USA). The certified surface density of amino groups is as high as 92 μmol/ml. Affigel 102 (Affigel) was purchased from Bio-Rad. The certified surface density of amino groups is as high as 17 μmol/ml. Seventy-six percent *w*/*w* phenol/EtOH (phenol/EtOH) was purchased from Applied Biosystems (Tokyo, Japan).

## Devices

UV-mini 1240 UV-VIS spectrophotometer (SHIMADZU, Kyoto, Japan), E-1010 Ion Spattering Apparatus (HITACHI), and Miniscope TM-1000 (HITACHI) were used.

## Titration method

A 0.1 N NaOH solution was approved by a standard 0.1 N oxalic acid aqueous solution prepared by ourselves using a standard method. A 0.1 N HCl solution was then standardized by the 0.1 N NaOH standard solution.

**Table 1** Feed composition of Moli-gel

| Symbols | Lig-m (μl; ratio of mole) | DEG-m (μl) | 9G (μl) | DEG-p (μl) | ADVN (mg) |
|---|---|---|---|---|---|
| L-0.1 | 3.5 (0.1) | | | | |
| L-0.5 | 17.4 (0.5) | | | | |
| L-0.75 | 25.9 (0.75) | | | | |
| L-1 | 34.5 (1) | 15.8 | 390.8 | 750.0 | 10.0 |
| L-2 | 68.9 (2) | | | | |
| L-3.5 | 120.8 (3.5) | | | | |
| L-5 | 172.3 (5) | | | | |

**Table 2** Relative residual error and error range in determination of amino group density by titration method

| Item | Titration method | |
|---|---|---|
| | Relative residual error (%) | Error range of amino group density (μmol/ml) |
| Octylamine | 0.1 | 0.1 |
| Dodecylamine | – | – |
| 3-Phenyl-1propylamine | – | – |
| Affigel | 0.2 | 0.0 |
| Toyopearl | 2.7 | 2.5 |

### *Titration of octylamine*

Octylamine solution of 68.8 μmol/ml was prepared by octylamine and EtOH. Of this solution, 2.0 ml was admixed with 40.0 ml of pure water and the resulting solution was stirred. Then, 3.0 ml of the 0.1 N HCl was added and stirred again. This solution was titrated with the standard 0.1 N NaOH aqueous solution with phenolphthalein as the indicator by microburette.

### *Titration of solid supports*

Aqueous dispersion of 1.5–1.8 ml of Affigel or Toyopearl was poured into a graduated vial to measure the accurate volume of the solid supports after their sedimentation. Solid supports, 2.0 ml (for Affigel) and 3.0 ml (for Toyopearl), were reacted with the 0.1 N aqueous HCl for 24 h with stirring. After removal and recovery of resulting aqueous HCl by filtration, the solid supports were washed by pure water and the water was combined with the recovered HCl solution. This HCl solution was titrated with the standard 0.1 N NaOH aqueous solution with phenolphthalein as the indicator. Concentration of octylamine solution and amino group density of solid supports were calculated by the measured values.

## Ninhydrin method

### *Preparation of primary amine solutions for preparation of calibration curve*

Seven concentrations (137.6, 103.2, 68.8, 34.4, 11.5, 5.7, and 2.8 μmol/ml) of primary amine solutions were prepared using octylamine, dodecylamine, and 3-phenyl-1-propylamine.

**Table 3** Titration value and measured amino group density

| Solid phases or amine solution | Titration | *v* (volume of solid phases or amine solution; ml) | Titration value (ml) | $V_{HCl}$ (ml) | *X* (N) | *Y* (μmol) | *Z* (measured amino group density; μmol/ml) | Concordance rate (%) |
|---|---|---|---|---|---|---|---|---|
| Affigel | B | 1.70 | 2.051 | 2.000 | 0.103 | −1.635 | −1.0 | 0 |
| | | 1.80 | 2.045 | 2.000 | 0.102 | −1.034 | −0.6 | |
| | Ave. | | 2.048 | | | | −0.8 | |
| Octylamine | B | 2.00 | 1.700 | 3.00 | 0.0567 | 135.382 | 67.7 | 99 |
| | | 2.00 | 1.696 | 3.00 | 0.0566 | 135.783 | 67.9 | |
| | Ave. | | 1.698 | | | | 67.8 | |
| Toyopearl | A | 1.80 | 1.472 | 3.00 | 0.0524 | 166.414 | 92.5 | 98 |
| | | 1.80 | 1.555 | 3.00 | 0.0553 | 157.554 | 87.5 | |
| | Ave. | | 1.514 | | | | 90.0 | |

Titration A: $f_{NaOH}$=1.0674, $f_{HCl}$=1.0785, $f_{oxalic\ acid}$=1.0002; titration B; $f_{NaOH}$=1.0013, $f_{HCl}$=1.0187, $f_{oxalic\ acid}$=1.0030; known amino group density (μmol/ml): Affigel 17, Toyopearl 92, Octylamine 68.8

*Optimization of reaction time with primary amine solutions and plotting of the calibration curve using octylamine solution*

Primary amine solution of 20 μl each and 5 μl of phenol/EtOH, 100 μl of pyridine, and 25 μl of ninhydrin/EtOH were admixed to start the reaction at 95 °C for 2–5 min. After the reaction, 870 μl of 20% aqueous ethanol was added into the reaction mixture. The resulting solution was diluted by eight or 16 times using 20% aqueous ethanol. The resulting colored solution was spectroscopically measured at 570 nm using the UV spectrometer.

The reaction time to show the highest value of UV absorbance was optimized. Each primary amine solution was reacted for the optimal time and measured several times to be evaluated again. Then, calibration curve was plotted by the results obtained by the use of octylamine solutions.

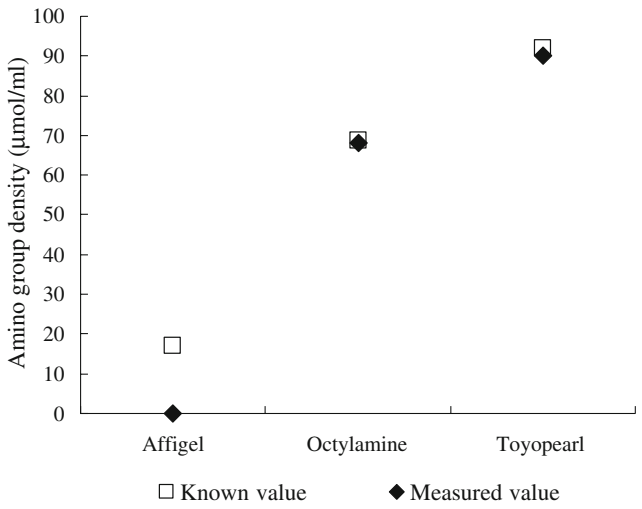


**Fig. 1** Relationship between certified amino group density and measured amino group density determined by titration method

*Optimization of reaction time for the solid supports and comparison with octylamine-based calibration curve*

The 20 μl aqueous dispersion of Affigel and Toyopearl was washed with 20% aqueous ethanol, then 5 μl of phenol/EtOH, 100 μl of pyridine, and 20 μl of ninhydrin/EtOH were added followed by the reaction at 95 °C for 3–10 min. Then, 870 μl of 20% aqueous ethanol was added into the reaction mixture to remove the "color" from the solid phases. This colored supernatant was diluted by two times again by 20% aqueous ethanol. The colored solution was spectroscopically measured at 570 nm using the UV spectrometer.

The optimal time to give the highest value of absorbance was studied before measurements. And each solid phase was reacted for the optimal time and measured several times. The measured values were compared with the octylamine calibration curve.

Preparation of Moli-gel (monolithic-type affinity gel) and calculation of theoretical amino group density and SEM observation of structure

As described in [1, 2], a standard monolithic-type affinity gel, namely Moli-gel, was prepared. In this case, Lig-m 186.4 mg (172.3 μl), DEG-m 16.6 mg (15.8 μl), 9G 435.7 mg (390.8 μl), DEG-p 844.7 mg (750.0 μl), and ADVN 10.0 mg were admixed in a vial at once and polymerized at 60 °C for 24 h. The resulting polymer was washed and comminuted, and then de-BOC reaction was carried out. Moli-gel with free amino group was then obtained.

To calculate the theoretical amino group density of the above prepared standard Moli-gel, 20 μl of Moli-gel (aqueous dispersion) was washed by 20% aqueous ethanol and dried (3.92 mg/20 μl). The amino group density of Moli-gel was measured by titration and

**Table 4** Relative residual error and error range in determination of amino group density by ninhydrin method

| Item | Ninhydrin method | | | Concordance rate (%) ninhydrin/titration |
|---|---|---|---|---|
| | Relative residual error (%) | Error range of amino group density calculated by octylamine calibration curve (μmol/ml) | Calculated amino group density by Octylamine calibration curve (μmol/ml) | |
| Octylamine | 2.1 | – | – | – |
| Dodecylamine | 3.9 | – | – | – |
| 3-Phenyl-1propylamine | 3.8 | – | – | – |
| Affigel | 4.9 | 4.4 | 22.4 | 132 |
| Toyopearl | 7.1 | 6.1 | 42.9 | 48 |

ninhydrin method using abovementioned processes. In the case of ninhydrin method, resulting colored supernatant was diluted by eight or 16 times using 20% aqueous ethanol. To discuss controllability of Moli-gel amino group density, Moli-gels were prepared using the prescribed feed composition summarized in Table 1. Scanning electron microscope observation took place after platinum–palladium ion spattering.

### Immobilization of ligand and calculation of immobilization rate

In the case of MTX as ligand, to prepare the solution of reagent, MTX, HOBt, and WSCD were dissolved into dry-NMP. Toyopearl or Moli-gel was washed by acetonitrile and dry-NMP. The prepared solution of MTX was added to 0.25 ml of Toyopearl or Moli-gel with 1.2 eq of WSCD and/or HOBt. The equivalent of added ligand was 0.5, 1.0 eq to the amino groups on each solid phase. The reaction was carried out at ambient temperature usually for 48 h. After the reaction, the amount of residual amino groups was determined using ninhydrin method in comparison to that of Toyopearl or Moli-gel without the immobilization; then, immobilization rate was calculated using absorbance as shown later.

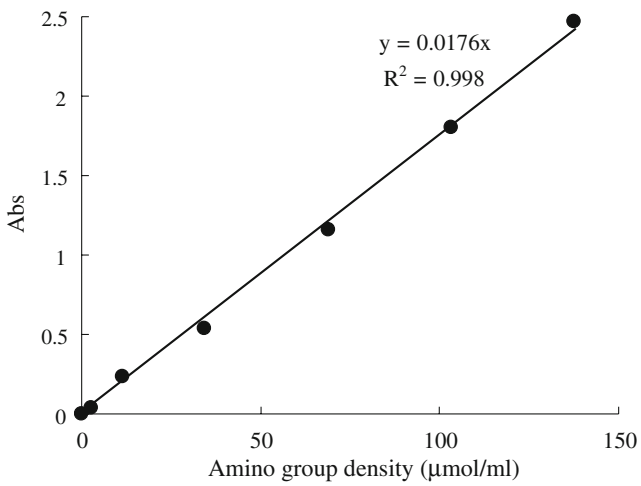


**Fig. 2** Octylamine calibration curve. Available range of this calibration curve was 1.7–139.0 μmol/ml of amino group density

## Result and discussion

### Quantitative evaluation of commercially available affinity resins having amino groups

In this study, we utilize two commercially available solid supports. One was Affigel that has 17 μmol/ml (water dispersion) of amino group density, while another was Toyopearl having 92 μmol/ml amino group density. Both densities were certified values from the manufacturers, while the detailed determination methods were not fully opened.

First of all, we selected octylamine was a standard compound of primary amine to calibrate and prove the titration method as well as ninhydrin method. For the

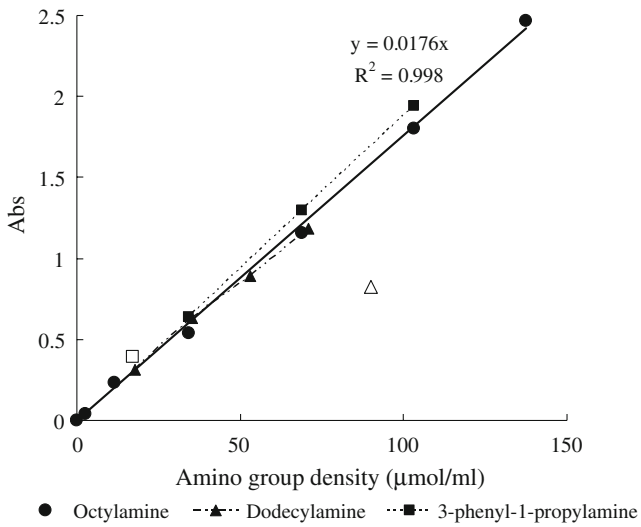


**Fig. 3** Validation of octylamine calibration curve using dodecyl amine and 3-phenyl-1-propylamine, and points of Affigel (*empty square*) and Toyopearl (*empty triangle*) showed Abs measured by ninhydrin method

**Table 5** Weight of each components and one repeat unit of polymer and theoretical amino group density about Moli-gel (L-5)

| | Lig-m | DEG-m | 9G | Weight of 1 unit of polymer (mg/μmol) | Theoretical amino group density (μmol/ml) |
|---|---|---|---|---|---|
| Ratio of mole | 5 | 1 | 9 | 7.275 | 134.0 |
| Weight of each components (mg/μmol) | 2.246 | 0.188 | 4.842 | | |

titration method, we prepared octylamine solution having 68.8 μmol/ml (theoretical value). Amino group density was calculated based on the following equation, where hydrochlorination to the amino group proceeded. Each item was admixed with 0.1 N HCl standard solution and residual HCl was titrated with 0.1 N NaOH standard aqueous solution.

If the concentration of the residual HCl is $X$ ($N$) and the volume of added 0.1 N HCl was $V_{\mathrm{HCl}}$ (ml),

$$X \times V_{\mathrm{HCl}}/1,000 = 0.1 \times f_{\mathrm{NaOH}} \times \text{used volume}/1,000 \quad (1)$$

$$X = (0.1 \times f_{\mathrm{NaOH}} \times \text{used volume})/V_{\mathrm{HCl}} \quad (2)$$

The amount of quaternized amino group was $Y$ (μmol),

$$Y(\mu\mathrm{mol}) = (0.1 \times f_{\mathrm{HCl}} - \mathrm{X}) \times V_{\mathrm{HCl}}/1,000 \times 10^{-6} \quad (3)$$

If calculated amino group density is $Z$ (μmol/ml) and the volume of used item solution is $v$ (ml),

$$Z(\mu\mathrm{mol/ml}) = Y/v \quad (4)$$

Table 2 proved the accuracy of the data by titration method. As shown in the table, relative residual error by titration method was smaller than 2.7%.

Table 3 and Fig. 1 showed the results by titration method including the densities of amino groups of Toyopearl and Affigel as well as the standard compound, octylamine. In addition, calculated concordance rates based on the certified values of Toyopearl and Affigel were shown in the table. In the case of the standard compound, octylamine, the concordance rate was calculated based on the exact value of prepared octylamine solution. Affigel unexpectedly afforded nearly 0 μmol/ml, while the reported value is 17 μmol/ml. On the other hand, the density of amino groups of Toyopearl was 90 μmol/ml. Since the reported value is 92 μmol/ml, the concordance rate was nicely 98%.

Ninhydrin method in this work was proved as shown in Table 4. Relative residual error using the standard compound, octylamine, was good enough (2.1%). Figure 2 proved the calibration curve prepared by a variety concentrations of octylamine. The calibration curve showed good linearity ($R^2$=0.998) up to 150 μmol/ml. We have proposed amino group densities of prescribed dodecylamine and 3-phenyl-1-propylamine solutions and plotted also on Fig. 3 with the calibration curve using the standard compound, octylamine. As shown in Fig. 3, excellent compatibilities were obtained for dodecylamine and 3-phenyl-1-propylamine, where relative residual error was smaller than 3.9% (Table 4).

We applied the proven ninhydrin method for the determination of amino group density of Affigel and Toyopearl. Table 4 also summarized those values including concordance rate based on the values obtained by titration method. As resulted before, the titration method afforded nearly 0 μmol/ml for Affigel; therefore, the concordance value of Affigel was calculated based on the certified value. In addition, Fig. 3 also indicated those values of Affigel and Toyopearl obtained by ninhydrin method where relative residual error was smaller than 7.1% (Table 4).

Unexpectedly again, ninhydrin method afforded nearly compatible amino group density for Affigel, where the concordance value based on the reported value was 132% (Table 4) and the value was nicely compatible to the calibration curve proposed using the standard compound,

**Table 6** Titration value and measured Moli-gel amino group density

| Solid phases | Titration | $v$ (volume of solid phases; ml) | Titration value (ml) | $V_{\mathrm{HCl}}$ (ml) | $X$ ($N$) | $Y$ (μmol) | $Z$ (measured amino group density; μmol/ml) | Concordance rate (%) |
|---|---|---|---|---|---|---|---|---|
| Moli-gel | A | 1.50 | 1.237 | 3.00 | 0.0440 | 191.499 | 127.7 | 94 |
| | | 1.50 | 1.297 | 3.00 | 0.0461 | 185.094 | 123.4 | |
| | Ave. | | 1.267 | | | | 125.5 | |

Titration A: $f_{\mathrm{NaOH}}$=1.0674, $f_{\mathrm{HCl}}$=1.0785, $f_{\mathrm{oxalic\ acid}}$=1.0002; theoretical amino group density: Moli-gel 134.0 μmol/ml

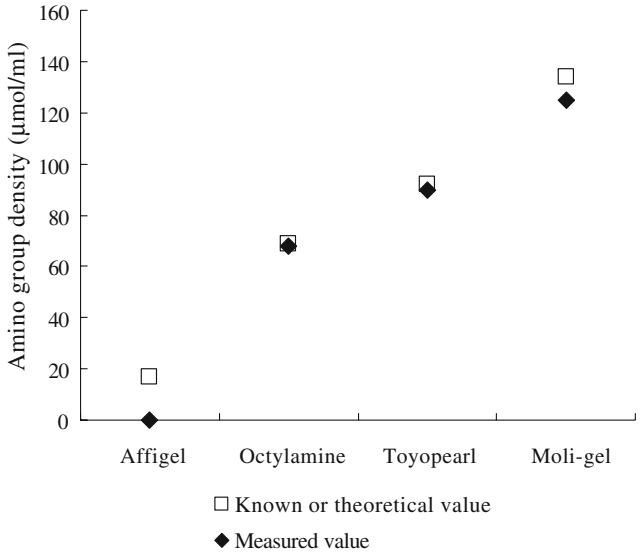


**Fig. 4** Relationship between known or theoretical amino group density and measured amino group density by titration method

octylamine. The incompatibility of Affigel between titration method and ninhydrin method might be explained based on chemical instability as well as existence of the other interferences for the titration.

On the contrary, in the case of Toyopearl, ninhydrin method unexpectedly afforded only 43 μmol/ml for Toyopearl, while the certified value is 92 μmol/ml. As plotted in Fig. 3, the value for Toyopearl was nearly half value of the calibration curve. As resulted before, titration method afforded nearly the certified value of Toyopearl; therefore, at this moment, the obtained incompatibility was only explainable due to existence of secondary amines or even higher amine because ninhydrin method is only applicable for primary amines. But the details are unclear at this moment because the detailed synthetic pathway has not been opened.

## Evaluations of newly prepared monolithic-type affinity support, namely Moli-gel

Considering the cross-incompatibilities observed in the previous section, we have to simplify the polymerization process as well as the monomers utilized to prepare the ideal affinity solid support. Based on our previous reports, we have prepared novel monolithic-type highly cross-linked affinity supports, namely Moli-gel. Moli-gel was just prepared by a simple copolymerization method using a special monomer having a protected amino group. We applied both the titration method and ninhydrin method to evaluate Moli-gel quantitatively.

First of all, we calculated "theoretical" amino group density based on the feed composition of the standard Moli-gel (L-5 in Table 1). As afforded in Table 5, the amino group density of one repeated unit of the copolymer was 0.687 μmol/mg. We dispersed 3.92 mg of this Moli-gel into 20 μl water in fact; therefore, the amino group density of the Moli-gel (L-5) dispersed in water was calculated as high as 134.0 μmol/ml.

Table 6 and Fig. 4 showed the results based on titration method, while the results obtained using ninhydrin method were summarized and plotted in Table 7 and Fig. 5, respectively. Table 7 proved accuracies of both methods for Moli-gel and those were acceptably good, where relative residual errors were smaller than 3.7%.

The titration method afforded 125.6 μmol/ml, which was comparable to 94% of the theoretical amino group density calculated in Table 5. In addition, ninhydrin method afforded 118.0 μmol/ml that was just comparable to 94% of the value observed by titration method. Ideally, both methods absolutely afford comparable amino group densities to each other if the polymer has only primary amine as the functional group on it. As described before, Moli-gel was just prepared by a copolymerization process using one kind of functional monomer having only a primary amine as the functional group; therefore, we were able to determine comparable amino group density using both of the titration and ninhydrin methods.

From the view point of preparation of affinity supports using copolymerization method, the obtained amino group density on Moli-gel will prove the nearly quantitative introduction of the amino monomers on the surface of polymer. This is quite interesting because, as mentioned in the "Introduction," usually copolymerization method will

**Table 7** Relative residual error and error range in determination of amino group density by titration and ninhydrin method for Moli-gel

| Solid phase | Items | | | | | Concordance rate (%) ninhydrin/titration |
|---|---|---|---|---|---|---|
| | Titration method | | Ninhydrin method | | | |
| | Relative residual error (%) | Error range of amino group density (μmol/ml) | Relative residual error (%) | Error range of amino group density calculated by octylamine calibration curve (μmol/ml) | Calculated amino group density by octylamine calibration curve (μmol/ml) | |
| Moli-gel | 2.4 | 3.2 | 3.7 | 4.4 | 118.0 | 94 |

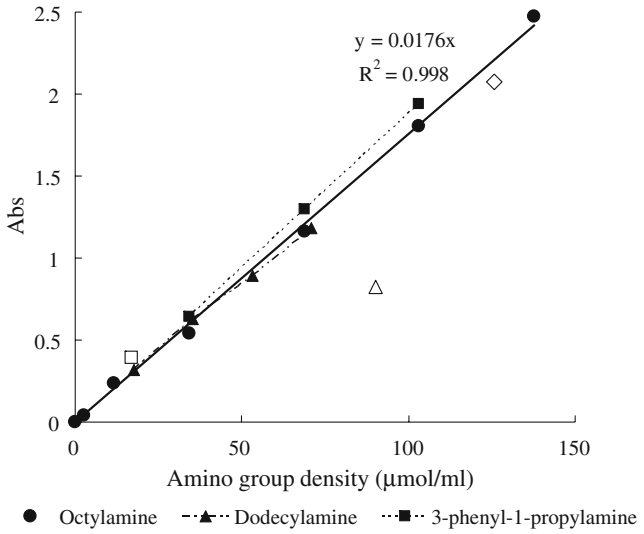


**Fig. 5** Validation of octylamine calibration curve using dodecyl amine and 3-phenyl-1-propylamine, and points of Affigel (*empty square*), Toyopearl (*empty triangle*), Moli-gel (*empty diamond*; L-5) showed Abs measured by ninhydrin method

lose functional monomers inside the cross-linked polymer matrices.

The amino group density of Moli-gel determined by ninhydrin method was comparable to the calibration curve using the standard compound, octylamine, as shown in Fig. 5; therefore, the easy and convenient determination method, ninhydrin method, is applicable to the determination of amino group density of Moli-gel. In addition, Moli-gel (L-5) has even higher amino group density compared to that of Toyopearl.

## Preparation and determination of amino group density of Moli-gels having a variety of compositions and observation of the structural characteristics

In the next step, we tried to prepare Moli-gels having a variety of composition, in other words, different amino group densities. Usually, copolymerization method using different monomer compositions is quite easy; however, in terms of monolith (cocontinuous structure) syntheses, a tiny difference in the monomer composition was reported to affect the polymer structure quite seriously. Therefore, observation of the structure is also very important for Moli-gel syntheses.

Table 1 showed the feed compositions as well as symbols of each Moli-gel. The amino group densities of each Moli-gel determined by ninhydrin method were plotted against the theoretical values as shown in Fig. 6. As expected, the observed values were nicely comparable to those calculated based on the feed composition of each Moli-gel. Although negligibly small errors were sill observed, it was proved that Moli-gel having a variety of amino group densities can be prepared with relatively wide range from 3 to 118 μmol/ml.

Figure 7 revealed scanning electron micrographs of each Moli-gel. Interestingly, all the Moli-gel somehow had monolithic structures (cocontinuous structures). As mentioned before, the monolithic structure is seriously affected by a tiny difference in monomer compositions, but, in the case of Moli-gel, monolithic structures were obtained with a rather wide range of monomer composition as shown in Table 1. This is a remarkable characteristic.

## Qualitative evaluation of Moli-gel and Toyopearl through immobilization reactions

The importance and/or convenience of solid supports utilized as affinity resins are principally dependent on reactivity and easily controlled coverage of the surface functional groups. Especially controlled immobilization of ligand is quite important because commercially available affinity resin has only one or two kinds of functional group density. We here quantitatively and qualitatively evaluated those characteristics through ligand immobilization reactions on commercially available Toyopearl. In this experiment, we utilized both the titration method and ninhydrin method to determine the surface coverage of the resulting Toyopearl.

First, we utilized monocarboxylic aids, sulfonamide and ketoprofen, as ligands. Those structures were illustrated in Fig. 8 (A) and (B). Here, 0.5 equivalent of each ligand to the certified amino group density of Toyopearl was reacted at ambient temperature with WSCD and HOBt. The

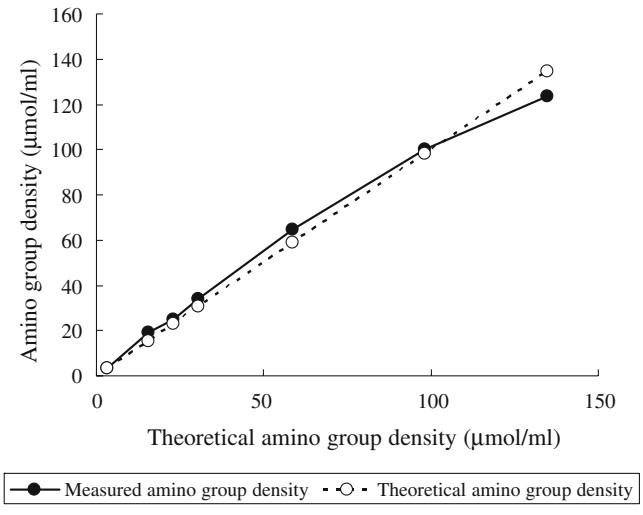


**Fig. 6** Relationship between theoretical amino group density and measured amino group density. Amino group density was calculated by ninhydrin method based on octylamine calibration curve

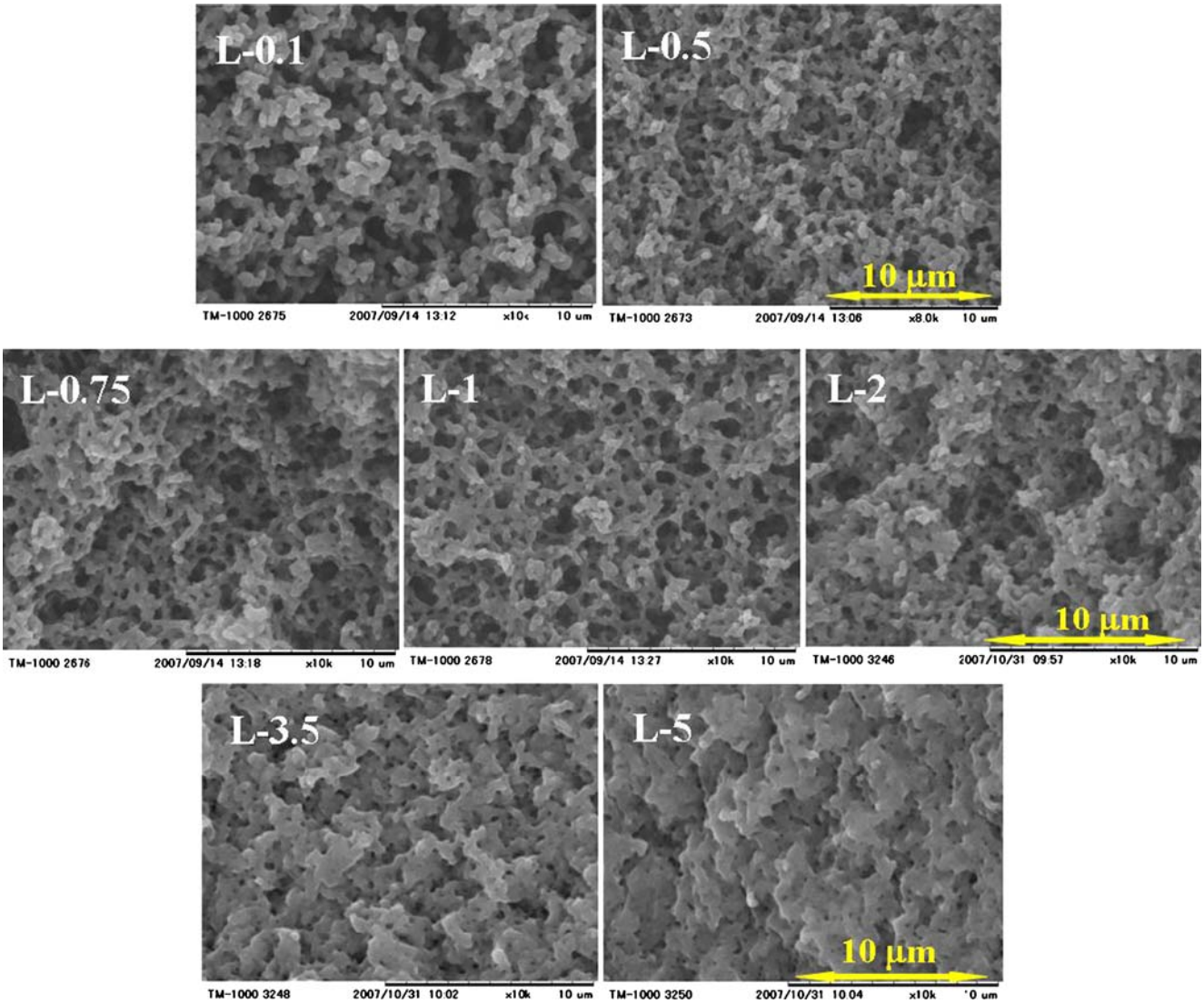


**Fig. 7** Scanning electron micrographs of Moli-gels having variety of amino group densities

immobilization rates were calculated based on the following equations.

For titration method:

$$\text{Immobilization rate}(\%) = (\text{reported amino group density} - \text{the amino})\text{group density after immobilization reaction} / \text{reported amino groupdensity} \times 100$$

For ninhydrin method:

$$\text{Immobilization rate}(\%) = (1 - \text{UV absorbance after reaction} / \text{UV absorbance before reaction}) \times 100$$

Figure 8 illustrated the results. As expected, titration method afforded 50% of immobilization rate to both ligands, while ninhydrin method showed rather higher value, 80% of immobilization rate. Since ninhydrin method can only determine the amount of primary amine, therefore, as speculated before, ninhydrin method was not able to afford the exact immobilization rate, while the immobilization reaction itself proceeded nicely and quantitatively.

Although titration method can be applicable for this measurement, in the case of affinity resin, ligand as well as affinity support materials tend to be expensive; therefore, titration method, where relatively large amount of affinity resins is required, is not convenient for this evaluation.

Secondly, we utilized relatively large dicarboxylic acid anticancer agent MTX as the ligand and 0.5 and 1.0 equivalents of MTX were reacted with Toyopearl and newly prepared monolithic affinity resin, Moli-gel (L-5). In this case, although MTX is dicarboxylic acid, the calculated

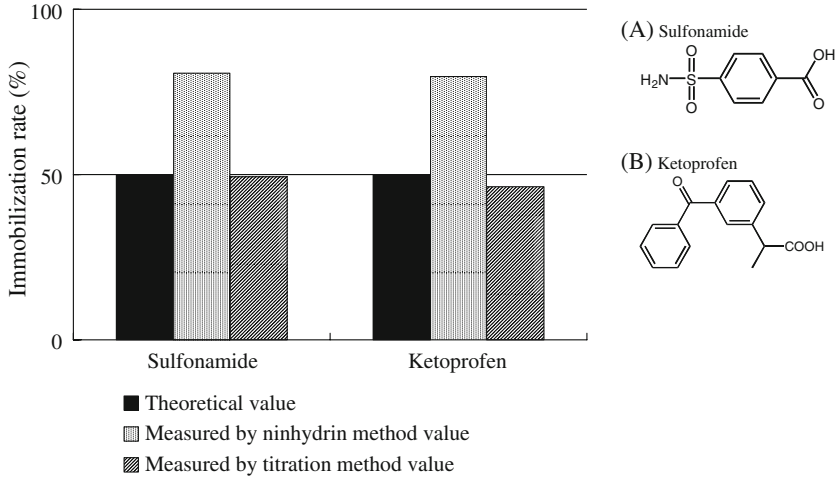


**Fig. 8** Sulfonamide and ketoprofen immobilization rate on Toyopearl measured by ninhydrin method and titration method. (*A*) and (*B*) are chemical structures of sulfonamide and ketoprofen, respectively

equivalent was based on monocarboxylic acid because just 1.0 equivalent of WSCD was utilized.

Figure 9 showed resulting immobilization rates determined by ninhydrin method. If 0.5 equivalent of MTX was reacted with Toyopearl, the determined immobilization rate was 67%, where 50% was the ideal immobilization rate. This rather higher immobilization rate was explained based on more consumption of primary amines on Toyopearl. On the other hand, 1.0 equivalent of MTX was utilized; the determined immobilization rate was only 74%, which was a much lower rate than that of the theoretically ideal rate, 100%. It was particularly worth nothing that ninhydrin method afforded lower immobilization rate. This observation suggested that primary amine still remained after the immobilization reaction even with 1.0 equivalent of MTX.

Moli-gel afforded 43% and 98% immobilization rates with 0.5 and 1.0 equivalents of MTX, respectively. These rates were nearly ideal immobilization rates on Moli-gel. In addition, further reaction with another carboxylic acid of MTX was proven to be negligibly small. Those observations strongly suggested that Moli-gel had qualitatively homogeneous amino groups which quantitatively reacted with ligands to result in the controlled immobilization rate. In addition, the immobilization rate was able to be determined by ninhydrin method, while it did not afford exact immobilization rate for Toyopearl.

## Conclusion

We have applied titration method and ninhydrin method to determine the density of surface amino group on polymeric solid supports. Both methods did not afford comparable amino density to commercially available solid supports, but newly prepared monolithic-type solid support was effectively determined by both the titration and ninhydrin methods. In addition, although copolymerization was employed for the preparation of monolithic solid supports, 94% of the functional monomer having a primary amino group was consequently introduced on the surface of solid support.

Monolithic solid supports, namely Moli-gel, were prepared with a variety of surface amino group densities without loss of monolithic structure. The introduced amino groups on Moli-gel were found to react with relatively large ligand with controlled immobilization rate and the immobilization rate was determined by easy and convenient

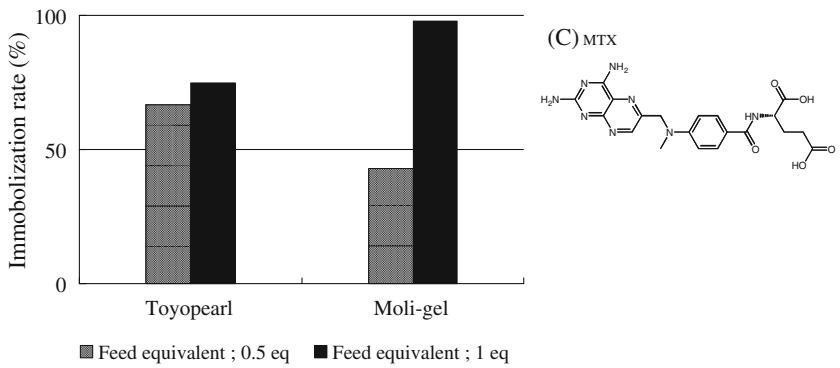


**Fig. 9** MTX immobilization rate on Toyopearl and Moli-gel measured by ninhydrin method. (*C*) is chemical structure of MTX

method, ninhydrin method. This phenomenon might prove qualitative homogeneous surface of Moli-gel and surface-concentrated introduction of amino groups on Moli-gels

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