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Click chemistry: A route to designing and preparing pseudo-biospecific immunoadsorbent for IgG adsorption

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ABSTRACT

L-histidine is a promising alternative to expensive protein ligands for the adsorption of IgG due to its high selectivity, no toxicity and low cost; while click chemistry can improve the reaction selectivity between the ligands and the support matrix under mild reaction conditions. Thus, using L-histidine as a ligand and original sepharose gel as a support, a novel immunoadsorbent possessing pseudo-biospecific affinity for IgG from human plasma, Sep-triazole-His, was designed and prepared according to the principle of Click-reaction between alkyne and azide functional groups; while both sepharose-based control samples Sep-His and Sep-PA were prepared by a conventional method using L-histidine and protein A as a ligand, respectively. The ligand density and IgG adsorption performance of Sep-triazole-His from human plasma were measured and evaluated. The influences of click chemistry on the preparation, structure and performance of sepharose-based immunoadsorbent were also investigated. The results indicate that the ligand density immobilized on Sep-triazole-His is 319.1 μmol/g sepharose gel, almost 4-fold as high as that on Sep-His; the IgG adsorption capacity of Sep-triazole-His from human plasma reaches 16.49 mg/g at pH 7.0, or increases 5.72-fold with respect to Sep-His, and does not decrease noticeably after being repeatedly used for 10 times; and Sep-triazole-His can exhibit high adsorption selectivity for IgG comparable to Sep-PA. The further studies prove that the 1,2,3-triazole ring in the spacer-arm of Sep-triazole-His, can facilitate the binding of IgG without non-specific adsorption.

1. Introduction

It has been known that proteins A, G, L, and anti-IgG ligands exhibit excellent biospecific affinity for immunoglobulin-G (IgG) [1,2], and the immunoadsorbents bearing these biospecific ligands have been proved to be effective for removing autoantibodies to treat severe forms of various autoimmune diseases [2] and purifying antibodies [3], thus being widely used in clinical practice and bioseparation. However, protein ligands possess some drawbacks that need to be taken seriously, such as potential immunogenicity, safety concerns caused by the detached protein ligands from the support matrix, sensitivity to harsh sanitization procedures, and often high cost [4–8]. Besides, protein ligands are difficult to be immobilized on the support matrix in the proper orientation [9]. To address some of these issues, the small and simple pseudo-biospecific ligands with high stability, excellent selectivity and low cost, such as amino acids, peptides, dyes, metal ions and the other synthetic ligands, have been developed, which can be promising alternatives to protein ligands [5–11]. Among them, L-histidine has been used as a pseudo-biospecific ligand instead of proteins in affinity chromatography due to its high selectivity, no toxicity and good reusability [12–16]. It has been reported that L-histidine shows particular efficacy in separating IgG from human plasma or serum [9,17–24].

In the conventional preparation of immunoadsorbents, ligands are covalently immobilized onto the support matrix bearing reactive groups such as amine, hydroxyl, carboxylic acid, aldehyde or thiol. However, complications can arise due to the lack of selectivity inherent in the aforementioned functionalities, which may result in ligands being bound in such a way to be detrimental to its affinity function when more than one of the attachment units is present in the ligand, or when other functional groups on the ligand compete with the desired coupling reaction [25]. Therefore, it is urgent and essential to improve the selectivity of coupling reaction between the ligand and the support matrix. A highly selective reaction between a pair of reactive groups attached to the ligand and the matrix, respectively, will be greatly helpful to optimize the preparation of immunoadsorbents. Yet click chemistry represents an attractive solution due to its high reliability, complete specificity and the biocompatibility of the reactants [25–28]. Click chemistry is a modular synthetic approach which is wide in scope, high yielding, stereospecific, simple to perform and uses benign solvent, creates only inoffensive by-products [27]. A premier and famous example of "click chemistry" is the Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of azides and alkynes to give triazoles. The mechanism of Cu(I)-catalysis for terminal azide–alkyne coupling

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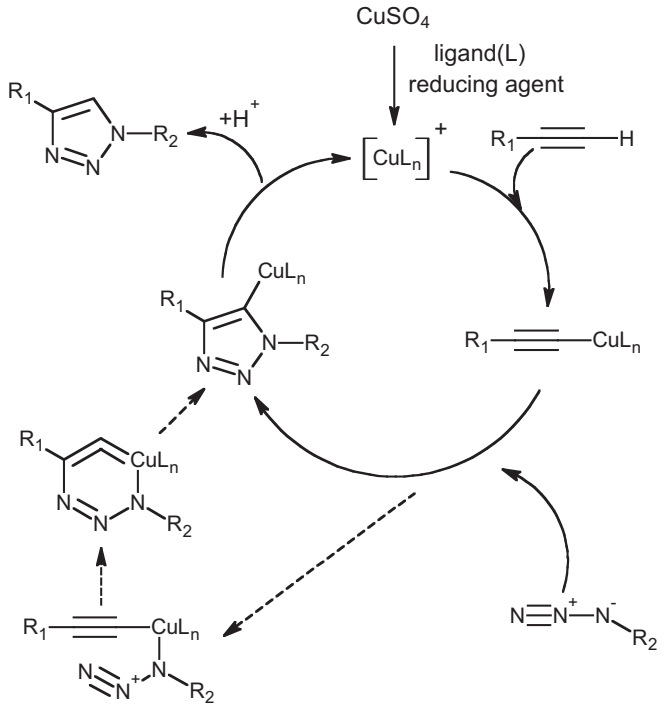


Fig. 1. The mechanism of Cu (I) catalysis for terminal azide–alkyne coupling reaction.

[29] is shown in Fig. 1. This click reaction is inert to most chemical functionalities and stable to wide ranges of solvent, temperature, and pH [29,30]. These features make click chemistry particularly attractive in bioconjugation, such as the development of sepharose-based affinity chromatography agents [31,32]. Based on this idea, sepharose beads bearing alkyne and azide groups were prepared from a commercial "specialty" sepharose–amine in order to conveniently immobilize ligands, and the as-prepared "clickable" sepharose beads can be successfully used as precursors to functional sepharose matrices for affinity chromatography [30]. Undoubtedly, it is of great significance to prepare similar "clickable" sepharose from sepharose itself and "clickable" ligands from much less expensive ligands for developing safe, efficient and less costly immunoadsorbents.

Therefore, this study will aim at exploring a novel route to designing and preparing pseudo-biospecific non-proteinous immunoadsorbent for IgG adsorption *via* click chemistry. For this purpose, an immunoadsorbent using L-histidine as a ligand and sepharose as a support was prepared *via* click chemistry. The adsorption performance of the prepared immunoadsorbent from human plasma were evaluated, and the influences of click chemistry on the preparation, structure and performance of sepharose-based immunoadsorbent were also investigated.

2. Experimental

2.1. Chemicals and materials

Sepharose 6FF (Sep) was purchased from Pharmacia (Sweden). Protein A was purchased from Vector Gene (Beijing). Plasma was provided by a hospital. L-Histidine, propargyl alcohol (99%) were purchased from Jingchun (Shanghai) and were used as received. Throughout this study only double-distilled water was used.

2.2. Instruments

2D ^{1}H NMR spectra were recorded on a 500 MHz NMR spectrometer (INOVA 500NB, Varian, American); the purity of IgG separated from plasma by Sep-triazole-His was determined by a MALDI-TOF mass spectrometer (ULTRAFLEX III, Bruker, Germany); UV spectra were obtained from an ultraviolet spectrophotometer (UV-2450, Shimadzu, Japan); FTIR spectra were performed on a FTIR spectrophotometer (Nexus Por Euro, Nicolet, American); elemental analysis were recorded on a Elemental Analyzer in CHNS Model (Vario EL III, elementar, Germany).

2.3. Preparation of immunoadsorbents

In order to prepare a novel immunoadsorbent *via* a highly selective click reaction of alkynes and azides, sepharose (Sep) as a support matrix was activated by epichlorohydrin followed by reacting with sodium azide to obtain azidated sepharose, Sep-N_3, as depicted in Fig. 2; and L-histidine as a small molecular ligand was functionalized to form L-histidine with terminal alkyne, His-alkyne, as described in Fig. 3. Then, the designed immunoadsorbent, Sep-triazole-His, could be prepared by the reaction between both clickable reactive modules Sep-N_3 and His-alkyne to yield triazole, as shown in Fig. 4. The specific steps (Figs. 2–4) were described in the electronic supplementary materials.

Furthermore, as a control sample for evaluating the efficacy of ligand L-histidine attached to Sep-triazole-His, a sepharose-based modified support matrix, Sep-triazole, was prepared *via* the click reaction of Sep-N_3 and propargyl alcohol as shown in Fig. 5.

For purpose of comparison, protein A immunoadsorbent using the same sepharose as a support, Sep-PA, was also prepared as given in detail in our previous work [33]. To evaluate the effectiveness of click chemistry, an immunoadsorbent (designated as Sep-His) was prepared by the same method as Sep-PA, the structure of Sep-PA and Sep-His were shown in Fig. 6.

2.4. Evaluation of the adsorption performance of Sep-triazole-His

2.4.1. IgG adsorption capacity from human plasma

The IgG adsorption capacity of Sep-triazole-His was measured by static adsorption experiments in a batch system. The human plasma was thawed for 1 h at 37 °C prior to use and the prepared immunoadsorbent Sep-triazole-His was equilibrated with 0.02 M

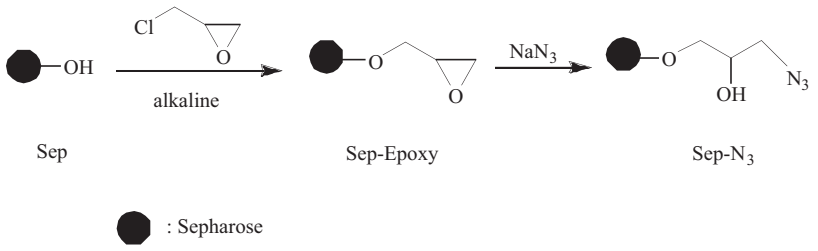


Fig. 2. The route to synthesizing of azidated sepharose (Sep-N_3).

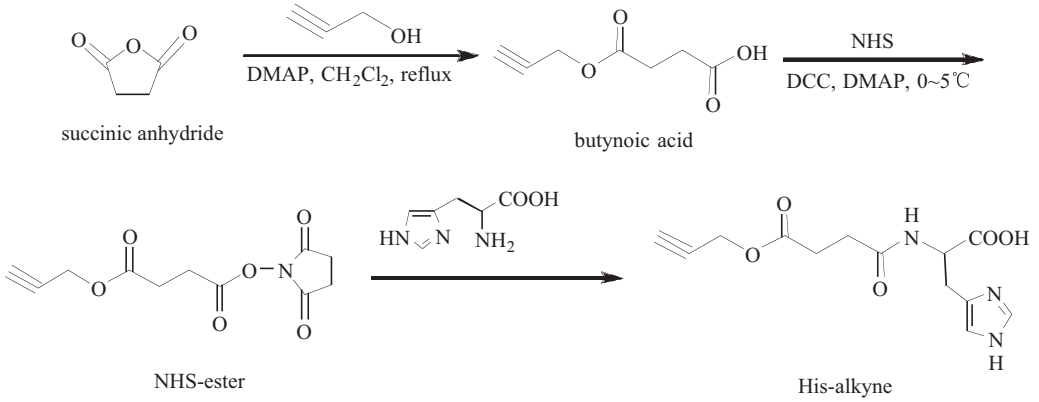


Fig. 3. The route to preparing the histidine with terminal alkyne (His-alkyne).

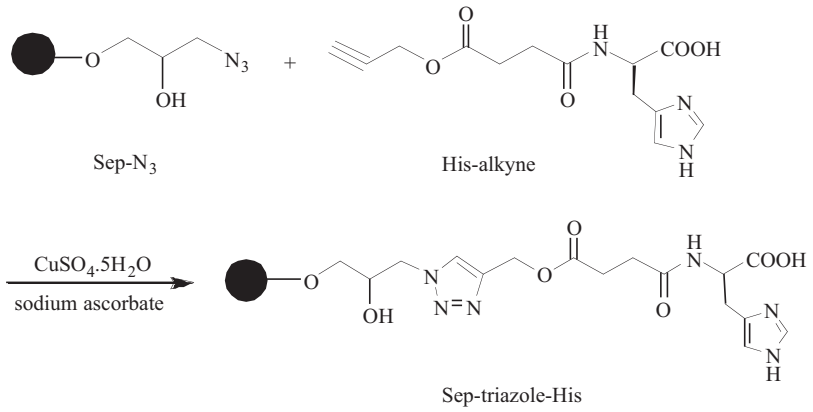


Fig. 4. The preparation of Sep-triazole-His by click chemistry.

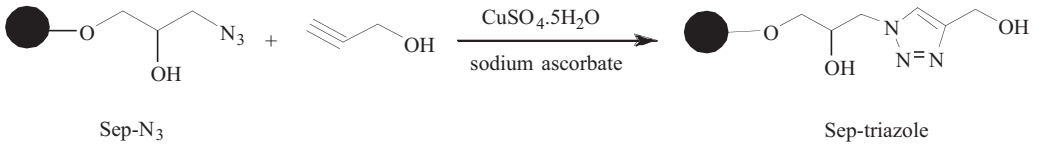


Fig. 5. The preparation and structure of Sep-triazole.

PBS buffer at pH 7.0 before being added to the human plasma. The static adsorption experiments were conducted at 25 °C and at a stirring rate of 100 rpm for 2 h. The Sep-triazole-His was suction-dried, the adsorbed IgG by the immunoadsorbent was eluted with 0.02 M citric acid buffer at pH 2.5. IgG concentration was determined by measuring the absorbance of the eluate at 280 nm (A_{280}) according to the Lambert–Beer law. The molar absorptivity of IgG is 1.35 ml/mg cm [34]. Then the amount of adsorbed IgG can be calculated.

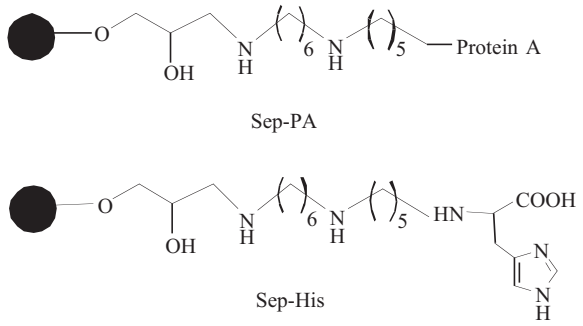


Fig. 6. The structures of Sep-PA and Sep-His prepared by a conventional method.

2.4.2. Adsorption selectivity of IgG from human plasma

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was used to analyze the adsorption selectivity of Sep-triazole-His for IgG from human plasma. The concentration of separation gel was 15% Tris–HCl, whereas stacking gel was 5% Tris–HCl. Samples were mixed in a 1:1 ratio with loading buffer and incubated for 5 min at 100 °C. The loading sample and molecular weight marker was 10 μL, staining with Coomassise brilliant blue R-250. And the purity of IgG separated by Sep-triazole-His from human plasma was further analyzed by MALDI-TOF MS in the positive ion mode using 2,5-dihydroxybenzoic acid (DHB) as a matrix.

2.4.3. Repeated use study

To evaluate the reusability of Sep-triazole-His, a series of 10 adsorption cycles of IgG from human plasma were performed as described above. Desorption test was achieved with 0.02 M citric acid buffer at pH 2.5, and then re-equilibrated with 0.02 M PBS buffer at pH 7.0, IgG binding capacity was measured for each cycle.

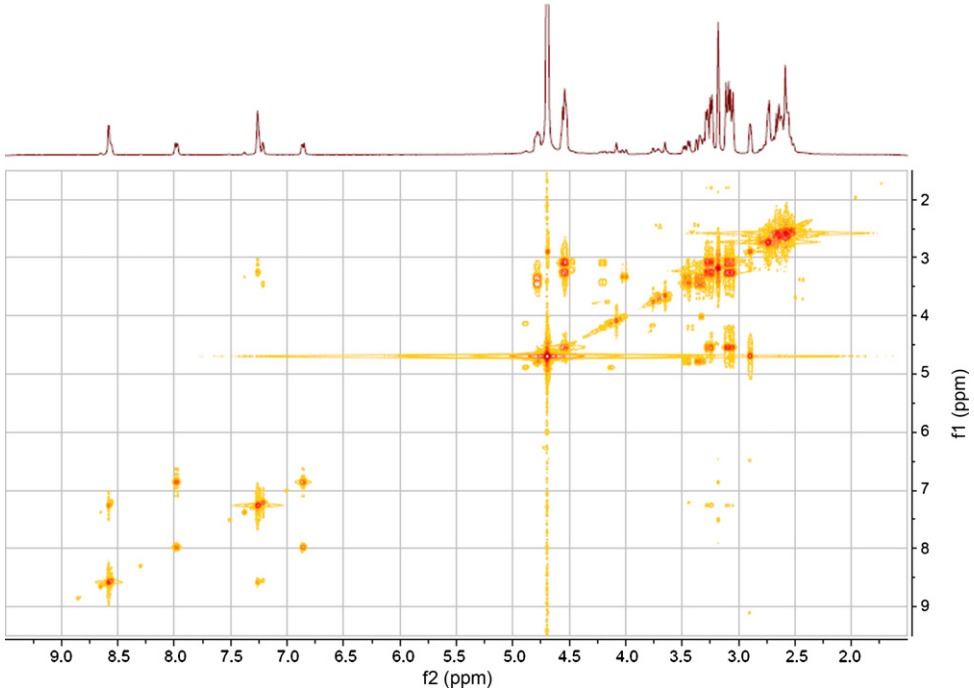


Fig. 7. The ^{1}H–^{1}H COSY spectrum of His-alkyne.

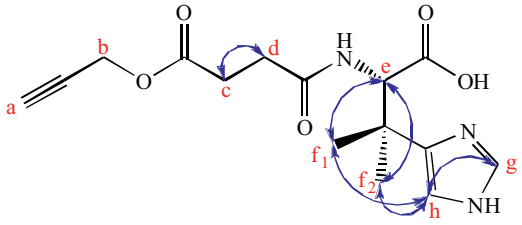


Fig. 8. Important ^{1}H–^{1}H COSY correlations of His-alkyne (↔).

3. Results and discussion

3.1. Structure characterization of the prepared products

3.1.1. Structural analysis of His-alkyne

To examine whether the ligand L-histidine was functionalized with terminal alkyne, the structure of the product His-alkyne prepared through the reaction steps described in Fig. 3 was analyzed by 2D ^{1}H NMR spectrum (^{1}H–^{1}H COSY) in D_2O, as shown in Fig. 7, and the spectrum data were listed in Table 1. Furthermore, important ^{1}H–^{1}H COSY correlations of His-alkyne were indicated by "↔" in Fig. 8.

The ^{1}H–^{1}H COSY analyses display the presence of alkyne, methylene and imidazole groups, which indicates that the structure of the product agrees well with that predicted by the click reaction theory.

Table 1
The ^{1}H–^{1}H COSY data of His-alkyne.

Position	δ_H (ppm)	Number of H	Peak shape	J (Hz)
g	8.58	1	m	1.20
h	7.26	1	m	0.68, 1.20
b	4.78	2	s	–
e	4.55	1	d d	8.40, 4.92
f1	3.24	1	d d	4.92, 0.68
f2	3.09	1	d d	8.40, 1.20
d	2.90, 2.73	2	m	4.72, 6.56
a	2.64	1	s	–
c	2.58	2	m	6.56, 4.80

3.1.2. Structure characterization of Sep-triazole-His

The available epoxy group content of epoxidized sepharose, Sep-Epoxy, was found to be 771.5 μmol/g gel by means of the acid–base titration; the azide content in Sep-N_3 was calculated to be 751.4 μmol/g gel according to the elemental analysis data; and the L-histidine ligand in Sep-triazole-His was 319.1 μmol/g.

The FTIR spectra of Sep, Sep-N_3 and Sep-triazole-His are presented in Fig. 9. Compared to the FTIR spectrum of Sep (Fig. 9(a)), the FTIR spectrum of Sep-N_3 (Fig. 9(b)) shows a sharp peak at 2100 cm^{-1} that is attributed to azide group, thus confirming that the sepharose has been functionalized with azide successfully. However, the peak at 2100 cm^{-1} dose not appear in the FTIR spectrum of Sep-triazole-His (Fig. 9(c)). This implies that the azide groups of Sep-N_3 have been consumed completely in the course of click reaction of Sep-N_3 and His-alkyne, which clearly exhibiting the high efficiency and selectivity of click chemistry.

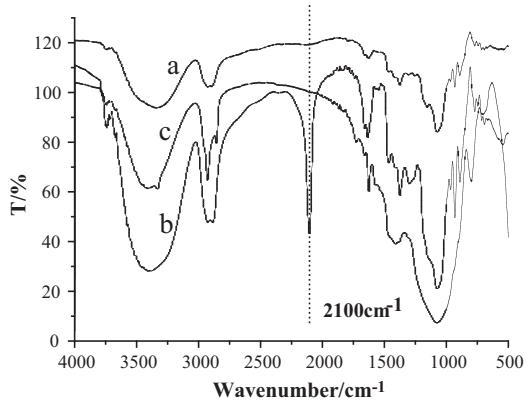


Fig. 9. The FTIR spectra of (a) Sep, (b) Sep-N_3 and (c) Sep-triazole-His.

3.2. Performance evaluation of Sep-triazole-His for IgG

3.2.1. Adsorption capacity of IgG from human plasma

As reported previously, the optimal pH value for adsorption of IgG can be usually observed at its isoelectric point (p*I*) [35], which is 6.95. For this reason, the adsorption of IgG onto the immunoadsorbents from human plasma was studied by the static adsorption experiment at pH 7.0.

The IgG adsorption capacity of Sep-triazole-His and Sep-triazole free of L-histidine was determined to be 16.49 and 2.97 mg/g, respectively. It indicates that the ligand L-histidine immobilized on Sep-triazole-His has the affinity for IgG. Moreover, carboxyl, amino and imidazole groups in the molecules of L-histidine can contribute to hydrophobic and electrostatic interaction with protein molecules [17]. Thus, IgG may be adsorbed onto L-histidine availably through these specific interactions.

The experimental results also show that the IgG adsorption capacity of Sep-triazole-His is lower than that of Sep-PA (22.97 mg/g). Apart from the structural differences of the spacer-arms of both immunoadsorbents, one reason for it may be that the macromolecular ligand protein A immobilized on Sep-PA contains multiple binding sites that are capable of interacting with the Fc region from IgG of several species [36,37].

3.2.2. Selectivity for IgG adsorption from human plasma

The purity of the adsorbed molecules in the eluates from the sample Sep-triazole-His and the control sample Sep-PA used in the IgG adsorption test was analyzed by SDS–PAGE, respectively, so as to evaluate their selectivity for IgG adsorption from human plasma. The result is presented in Fig. 10

As seen in Fig. 10, the bands corresponding to the light chain (25 kDa) and the heavy chain (50 kDa) of IgG can be observed in the eluates from both Sep-PA and Sep-triazole-His (Lanes 2 and 3). Besides IgG, traces of several other proteins are faintly visible in the eluate from Sep-PA but invisible in the eluate from Sep-triazole-His. It means that Sep-triazole-His possesses the ability of highly selective adsorption for IgG, and non-specific adsorption from human plasma is almost negligible. The result can be confirmed again by the MALDI-TOF MS analysis of the same eluate from Sep-triazole-His. As shown in Fig. 11, only the peaks at the mass-to-charge (*m*/*z*) ratio of 24,779 and 49,623, which are the characteristic peaks of IgG with different charge [38], can be clearly observed. It means that the designed and prepared immunoadsorbent Sep-triazole-His possesses the ability of selectively separating IgG from human plasma.

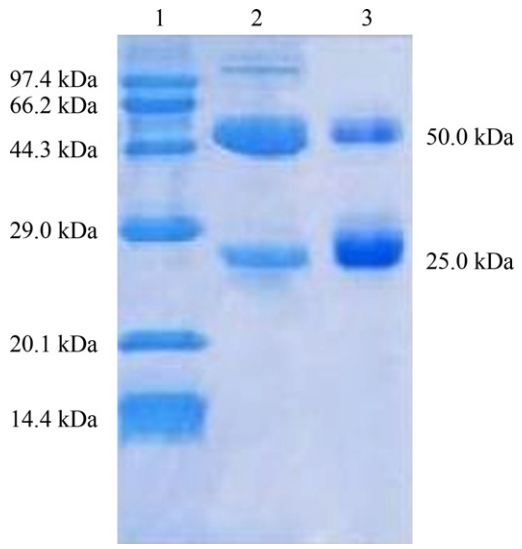


Fig. 10. SDS–PAGE analysis of fractions eluted from Sep-triazole-His and Sep-PA. Lane 1: protein markers; Lane 2: eluate from Sep-PA; Lane 3: eluate from Sep-triazole-His.

3.2.3. Adsorption stability over repeated use

The adsorption–desorption cycle was repeated 10 times by using the same immunoadsorbent sample to investigate its reusability. The data on IgG adsorption capacity with the times of reuse are listed in Table 2. It can be observed that IgG adsorption capacity declines slowly from the initial value of 16.49–14.52 mg/g during 10 cycles of usage, which means that Sep-triazole-His can be repeatedly used without noticeable loss in its IgG adsorption capacity to a certain extent.

3.3. Influences of click chemistry on the preparation, structure and performance of sepharose-based immunoadsorbent

3.3.1. Influence of click chemistry on the immobilization of ligand

As a novel method for preparing immunoadsorbents, the effect of click chemistry should be evaluated. For comparison, an immunoadsorbent Sep-His prepared using the same ligand by a conventional method was used as a control sample. The ligand density on Sep-His was 80.2 μmol/g, which was also determined by elemental analysis. However, as given above, the ligand density on Sep-triazole-His prepared *via* click chemistry was 319.1 μmol/g, which is almost 4-fold as high as that on Sep-His.

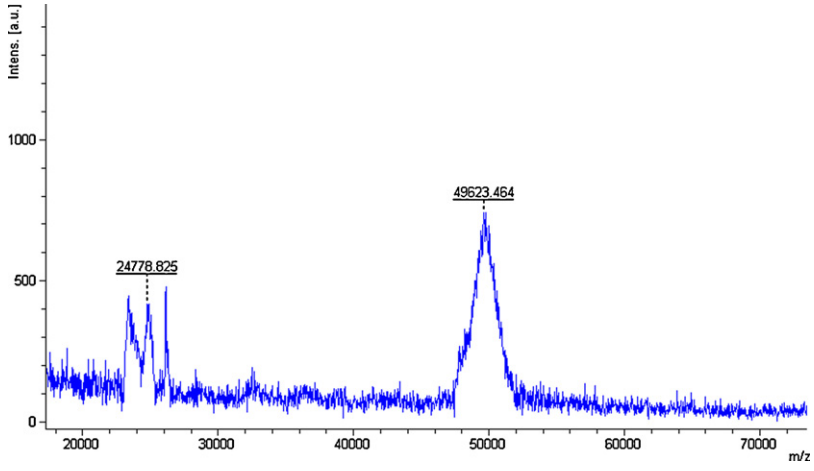


Fig. 11. Analysis of fractions eluted from Sep-triazole-His by MALDI-TOF MS.

Table 2
Adsorption stability over repeated use of Sep-triazole-His.

Times of reuse	1	2	3	4	5	6	7	8	9	10
IgG adsorption capacity (mg/g)	16.49	16.02	16.17	15.59	15.75	15.19	14.95	14.66	14.80	14.52

It is clear that the click reaction of the azidated support, Sep-N_3, with the alkyne-functionalized ligand, His-alkyne, can greatly promote the immobilization of ligand onto the support matrix, so significantly increasing the density of ligand covalently linked to the sepharose. The reason is that an alkyne and an azide moiety, a matched pair of reactive groups, can couple with each other to yield 1,2,3-triazole ring, which is in essence highly selective, fast and straight forward, and stable to wide ranges of reaction conditions [30,31], so that no side reaction can occur. As a highly selective click reaction between both "clickable" reactants, the Cu(I)-catalyzed azide–alkyne cycloaddition between Sep-N_3 and His-alkyne, can inhibit undesired reactions from occurring to a considerable extent, thus increasing the amount of ligands immobilized on the support in an expected way. Therefore, click chemistry is very useful to improve the selectivity between a ligand and a support matrix and increase the immobilization efficiency of ligands.

3.3.2. Analyses of the structure and performance of sepharose-based immunoadsorbents

Besides the ligand density, both sepharose-based immunoadsorbents Sep-triazole-His and Sep-His prepared by click chemistry and a conventional method, respectively, possess different structural spacer-arms, which can influence their adsorption performance. In order to conveniently compare and analyze the structural difference of spacer-arm and its influence on the adsorption performance, the important structural characteristics and the IgG adsorption capacity of Sep-His, Sep-triazole-His, the corresponding modified sepharose support bearing no ligand, Sep-triazole, and a bare sepharose, Sep, are listed in Table 3.

As seen in Table 3, a bare sepharose with no spacer-arm and no ligand attached nearly has no adsorption for IgG (0.24 mg/g); the IgG adsorption capacity of Sep-triazole-His is 16.49 mg/g, or increases 5.72-fold with respect to Sep-His, while the ligand density on Sep-triazole-His is about 4-fold as high as that on Sep-His. Unexpectedly, the control sample, Sep-triazole with no ligand attached, shows affinity for IgG to a certain extent, and its IgG adsorption capacity (2.97 mg/g) is basically equal to that of Sep-His (2.88 mg/g). These results suggest that the high ligand density can be not the only reason for an increase in the IgG adsorption capacity of Sep-triazole-His. Obviously, the structural difference of spacer-arms, through which the ligand L-histidine and the support Sep are covalently linked together, should be an important factor influencing the adsorption performance of the prepared immunoadsorbents Sep-triazole-His and Sep-His.

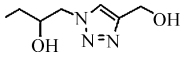
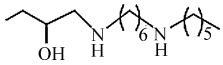
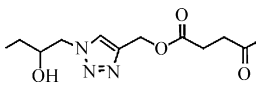
In general, if a ligand is coupled directly to the polymeric support, steric hindrance with the ligand interaction will occur [11]. Therefore, an appropriately designed spacer-arm placed between the support and the ligand is often bound to the support prior to immobilizing the ligand [17]. By this way, the steric hindrance between the support surface and the target biomolecules can be prevented, thus facilitating the small ligands interacting with the target biomolecules.

From the comparison of spacer-arms of both Sep-triazole-His and Sep-His shown in Table 3, it can be seen that the former is relatively shorter than the latter and contains the 1,2,3-triazole ring produced by an azide–alkyne cycloaddition, while the latter does not. The presence of 1,2,3-triazole ring can make the spacer-arm of Sep-triazole-His more rigid than that of Sep-His. Theoretically, the long spacer-arm should be more effective to decrease the steric hindrance than the short one. However, a long and flexible spacer-arm may partly lie on the support surface, which may make some of the binding sites of ligands shielded, so not working. Based on these analyses, it may be reasonably said that the spacer-arm containing 1,2,3-triazole ring in Sep-triazole-His possesses a suitable rigidity, thus preventing the steric hindrance and increasing the accessibility of L-histidine ligand to IgG.

Since the triazole group in the spacer-arm can exhibit affinity for IgG from human plasma, it is of importance to clarify whether it can cause non-specific adsorption or not. Therefore, the adsorption selectivity of the modified sepharose support, Sep-triazole, was also examined by SDS–PAGE method. For the convenience of comparison, the experimental results of Sep, Sep-triazole, Sep-His and Sep-triazole-His obtained by SDS–PAGE are shown in Fig. 12. It can be noticed that except the bands corresponding to the light chain (25 kDa) and the heavy chain (50 kDa) of IgG, no traces of other proteins are observed in the eluate from Sep-triazole, which is similar to the corresponding test results obtained from the eluates of Sep-His and Sep-triazole-His. It indicates that the triazole ring in the spacer-arm of Sep-triazole-His can facilitate the binding of IgG without non-specific adsorption.

It is obvious that the triazole ring possesses nitrogen atoms that might act as hydrogen bond acceptors. The studies on the 1,2,3-triazole ε^2-amino acid as a dipeptide surrogate in α-helical coiled coil have proved that the triazole ring can participate in the backbone hydrogen bonding of the α-helix [39]. On the other hand, the triazole ring has a large dipole that could align with that of the other amides in a given peptide secondary structure [40]. It suggests that the triazole ring in the spacer-arm can interact with the different active sites of IgG and form some ordered structure like helix *via* hydrogen-bonding and dipole forces. In fact, there exist multi-interactions in the adsorption process of IgG on immunoadsorbents [11,17,36]. Therefore, both interactions

Table 3
The structural characteristics of sepharose-based immunoadsorbents having different spacer-arms and their IgG adsorption performance from human plasma.

Sample	Structure of spacer-arm	Ligand density (μmol/g)	IgG adsorption capacity (mg/g)
Sep	–	–	0.24
Sep-triazole		–	2.97
Sep-His		80.2	2.88
Sep-triazole-His		319.1	16.49

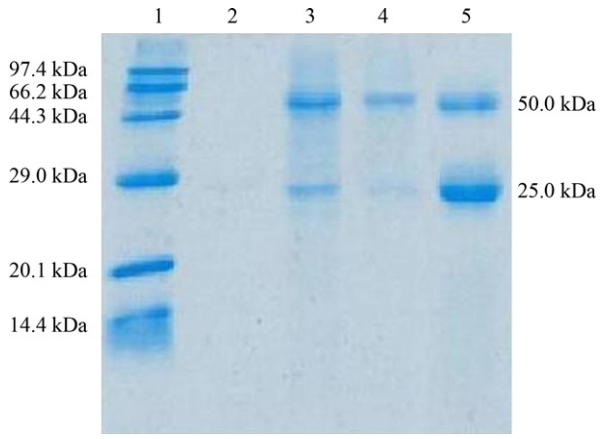


Fig. 12. SDS–PAGE analysis of fractions eluted from sepharose-based immunoadsorbents. Lane 1: protein markers; Lane 2: eluate from Sep; Lane 3: eluate from Sep-triazole; Lane 4: eluate from Sep-His; Lane 5: eluate from Sep-triazole-His.

between the triazole ring and IgG molecule may differ from those between the ligand L-histidine and IgG molecule, but also can contribute to the non-covalent combination of IgG molecules with immunoadsorbents as a complementary role. This is the reason for that Sep-triazole-His has higher IgG adsorption capacity than Sep-His.

Besides, it can be assumed from the above studies that the geometry and conformation of the spacer-arm containing triazole ring may be helpful to form ordered structures with IgG molecules through some specific binding. This inference may account for the high adsorption selectivity of Sep-triazole-His for IgG from human plasma. To confirm this, the meticulous research is required.

4. Conclusions

In this study, click chemistry was adopted as a novel route to designing and preparing pseudo-biospecific non-proteinous immunoadsorbents using L-histidine as a ligand and original sepharose gel as a support. The ligand density immobilized on the as-prepared immunoadsorbent Sep-triazole-His is 319.1 μmol/g sepharose gel, almost 4-fold as high as that on the immunoadsorbent Sep-His prepared by a conventional method; the IgG adsorption capacity of Sep-triazole-His from human plasma reaches 16.49 mg/g at pH 7.0, or increases 5.72-fold with respect to Sep-His, and does not decrease noticeably after being repeatedly used for 10 times; and Sep-triazole-His can exhibit high adsorption selectivity for IgG comparable to the Protein A immunoadsorbent Sep-PA. Besides, the 1,2,3-triazole ring in the spacer-arm of Sep-triazole-His, which is produced *via* the click reaction between azide and alkyne functional groups, can facilitate the binding of IgG without non-specific adsorption.

Based on these findings, the click reaction can greatly increase the selectivity of the coupling reaction, thereby increase the immobilization efficiency of ligands. Thus, it may be safely said that click chemistry provides a novel feasible route to developing safe, efficient and less costly non-proteinous immunoadsorbents. The further research will focus on increasing the amount of ligand immobilized on the support matrix and optimizing the structure of spacer-arm so as to improve the IgG adsorption performance of pseudo-biospecific immunoadsorbent.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.05.007.

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