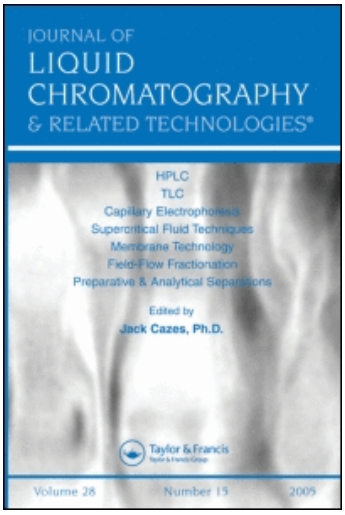

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A NOVEL METHOD FOR PREPARATION AND CHARACTERIZATION OF RESTRICTED-ACCESS MEDIA-ALKYL-DIOL SILICA (ADS)

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A NOVEL METHOD FOR PREPARATION AND CHARACTERIZATION OF RESTRICTED-ACCESS MEDIA-ALKYL-DIOL SILICA (ADS)

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ABSTRACT

A novel method had been developed to prepare ADS packings. Different alkyl-chains were bound onto Develosil60-5μ silica (particle size dp = 5 μm, s = 400-450m^2/g) with γ-glycidoxypropyltrimethoxysilane as coupling agent and hydrophilic diol phase precursor. Characterization of the packings was carried out with elemental analysis, X-Ray Photoelectron Spectroscopy (XPS), Fourier Transform Infrared (FT-IR), solid-state ^{13}C, and ^{29}Si CP-MAS NMR. Chromatographic tests confirm the essence of the packings and RAS behavior.

INTRODUCTION

Classical methods of sample preparation include precipitation and extraction for analysis of complex biological matrices in LC, which serves to eliminate

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interfering compounds and to remove analyte from the protein matrix, but these methods are time-consuming and labor-intensive and, furthermore, low recoveries was found.[1]

Direct injection of samples onto HPLC columns is substantially advantageous in terms of its time- and labor-saving capabilities. The methods include the precolumn technique, restricted-access media, and chromatography in mobile phases containing surfactant, and similar approaches.[1] An attractive method to quantify small molecules in complex biological matrices, such as plasma, and to eliminate the necessity for any sample pretreatment, is LC with column switching. In coupled-column LC, the target molecules are first dynamically extracted by a sample processing precolumn and subsequently separated on an analytical column. Conventional silica-based precolumn packing materials, however, usually still require a sample clean-up step, such as precipitation or dialysis prior to injection in order to remove the protein matrix. Otherwise, the protein accumulates on the packing of the precolumn, resulting in a decrease in column performance, an increased back-pressure, and finally, a limited number of analysis cycles.

Special chromatographic supports, used in single- and coupled-column mode, allowing the direct and repetitive injection of untreated proteinaceous fluids have been developed during the last few years.[2-4] These so-called restricted-access materials exclude macromolecules, such as plasma proteins without destructive accumulation, but retain small target molecules by partitioning or affinity chromatography.[5] With RAM Direct Injection HPLC columns, a variety of complex sample matrices can be injected directly, without prior sample clean-up, and drugs, drug metabolites, peptides, and other compounds can be separated and detected.

Alkyl-Diol Silica, which was a chemically and enzymatically modified packing material first introduced by Boos et. al.,[6] belongs to the group of restricted-access media having a physical diffusion barrier.[7-9] Its preparation includes three steps: (1) The hydrophilic diol groups were bonded to micropore silica; (2) The diol groups are reacted with fatty acid chlorides having different n-alkyl chain lengths (C4, C8, C18); (3) The packings are treated with pancreatic lipase and/or esterase to remove the hydrophobic partitioning moieties (fatty acids), exclusively, from the outer surface by enzymatic hydrolysis. The molecular mass cut-off of the modified packings is approximately 15 kDa.

This paper reports an improved method for the preparation of ADS. The proposed method also includes three steps: (1) The γ-Glycidoxpropyl trimethoxysilane (containing epoxy groups) was bonded onto 60Å Develosil60-5 μ silica; (2) Epoxide on silane was reacted with fatty acid having different n-alkyl chain lengths (C4, C8, C12, C16, C18) under the catalysis of boron trifluoride at high temperature; (3) The packings were treated with pancreatic lipase (EC

3.1.1.3) and/or esterase to remove the hydrophobic partioning moieties (fatty acids), exclusively, from the outer surface by enzymatic hydrolysis. The packings with long carbon-chains can also be used as RP packings, which will be discussed in another paper.

EXPERIMENTAL

Chemicals and Solvents

The silica utilized (mean particle diameter dp = 5 μm, surface area S=300 m^2/g) was supplied by Momura Chemical Co. Ltd., Japan. The Glycidoxypropyl trimethoxysilane was purchased from Yingkou Chemical Co., China. Stearic Acid (analytical-reagent grade, Beijing Chemicals factory, China), Acetonitrile (HPLC grade, Fisher Chemicals), Trifluoroacetic acid (Protein sequencing grade, SIGMA), Pancreatic lipase (E.C.3.1.1.3, SIGMA), Methanol (excellent reagents, Beijing chemicals factory), Bovine sodium cholate (Beijing Biological Products Institute). Other reagents utilized are all analytical-reagent grade.

Apparatus

For the HPLC analysis, separations were carried out at ambient temperature on a system comprised of a Beckmann Liquid-delivery system and sampling valve with 50 μL-loop, a syringe injector, 50 μL, a KARATOS Spectroflow 783 Programmable Absorbance Detector, a chart recorder (Hitach Ltd.), a mechanic Agitator (Digital 2000, Heidolph RZR-2000), a pneumatic amplification pump (Chemco Packer, Japan).

Synthetic Procedures

Bonding of Glycidoxypropyl Trimethoxysilane onto Silica

Dried silica gel was placed in a 250-mL three-neck round-bottom flask, then the flask was heated up to 150°C under vacuum for 4h. After this period, glycidoxypropyltrimethoxysilane and triethylamine, which were dissolved in dried toluene, were driven into the flask by the vacuum within. The slurry was agitated at refluxing temperature in an oil bath for 6-8h. The bonded phase support was filtered and washed with toluene and acetone, respectively. The support was then dried in vacuum at 60°C for 2h.

Preparation of C_{18}-Ester-Bond Reversed-Phase Packing

Dried support described above was placed in a 250-mL three-neck round-bottom flask and then was treated under vacuum for 4h. After that, the vacuum within drove the stearic acid, which was dissolved in decalin, into the flask. The slurry was agitated at high temperature 150°C for 6-8h. The bonded phase support was filtered and washed with decalin and acetone, respectively. The support was then dried in vacuum at 60°C for 2h.

Enzymatic Hydrolysis of the External Surfacial C_{18} Ester-Bond

The ester-bond RP phase packing was packed into a stainless steel column (ϕ4.0 × 50), then the hydrolytic solution (0.2%w/w, Tris-HCl buffer, pH7.5) of pancreatic lipase (EC 3.1.1.3), containing both bovine sodium cholate (0.4%w/w) and calcium (0.1%w/w, as activation agent) as mobile phase, was filled into the column. With enzymatic solution going through the column, the hydrophobic partioning moieties (fatty acids) were removed, exclusively, from the outer surface by enzymatic hydrolysis.

Product Evaluation and Characterization

Elemental Analysis and Spectroscopic Studies

Elemental analysis was performed on a ST-02 Elemental Analyser; Solid-state ^{13}C and ^{29}Si CP-MAS-NMR spectra were obtained on a Bruker Spectrometer; XPS spectra was determined on ESCALab220I-XL. FT-IR Spectrum was obtained on PE 2000 FT-IR Spectrometer.

Chromatographic Evaluation

The bonded phase packing was packed into a 50 × 4.0 I.D. stainless steel column using a pneumatic amplification pump at 40Mpa. Test solutes included benzene, naphthelene, phenanthrene, and some proteins.

RESULTS AND DISCUSSION

Elemental Analysis

After bonding of γ-glycidoxypropyltrimethoxysilane onto silica, the concentration of carbon was 8.4%; the carbon concentration of C_{18}-ester-bond

reversed-phase packing was 16.5%; the carbon concentration of RAS was 16.5%. From elemental analysis, the success of the first and second step can be confirmed, while whether or not the third step is successful can not be confirmed. This is due to the low outer surface area of the micropore spherical silica as compared to its internal surface.

Characterization of the Products with X-Ray Photoelectron Spectroscopy (XPS), Fourier Transform Infrared (FT-IR) and Solid-State ^{29}SI and ^{13}C CP-MAS-NMR

XPS, FT-IR, Solid-state ^{29}Si, and ^{13}C CP-MAS-NMR were applied to characterize the final product. XPS and Solid-state ^{13}C spectrum of the packing can confirm the success of the bonding reaction.

Characterization with XPS

The success of the preparation process can be confirmed with XPS spectrum (Figure 1, Figure 2) of the products.

As can be seen from Figure 1, the peak at 286.95ev (C1s, the first ionization energy of carbon) indicated the existence of carbon on the epoxide group: , the peak at 285.06ev (C1s) indicated the existence of carbon on methylene chain. The success of the first step (reaction between glycidoxypropyltrimethoxysilane and silica) can be confirmed, and also can be confirmed by elemental analysis described above.

Figure 2 is the FT-IR spectrum of the ester-bonded intermediate. To eliminate the interference from the silica support, the silica was used as background. As can be seen from Figure 2, the peak at 2926.08 cm^{-1} and 2856.27 cm^{-1} are asymmetric stretching vibration and symmetric stretching vibration of $–CH_2–$, respectively; and the peak at 1467.05 is also assigned to the bending vibration of $–CH_2–$, which indicated the existence of methylene. The peak at 1740.54 cm^{-1} is assigned to the stretching vibration of group C = O on the ester group, which indicated the existence of the ester-bond. So, Figure 2 can confirm the success of the second step (ring-opening reaction and coupling of C_{18} chain onto silica), and the results of elemental analysis can also confirm the success of the procedure.

Characterization with Solid-State ^{29}Si and ^{13}C CP-MAS NMR

The final product RAS was characterized with solid-state ^{29}Si and ^{13}C CP-MAS NMR (Figures 3, 4).

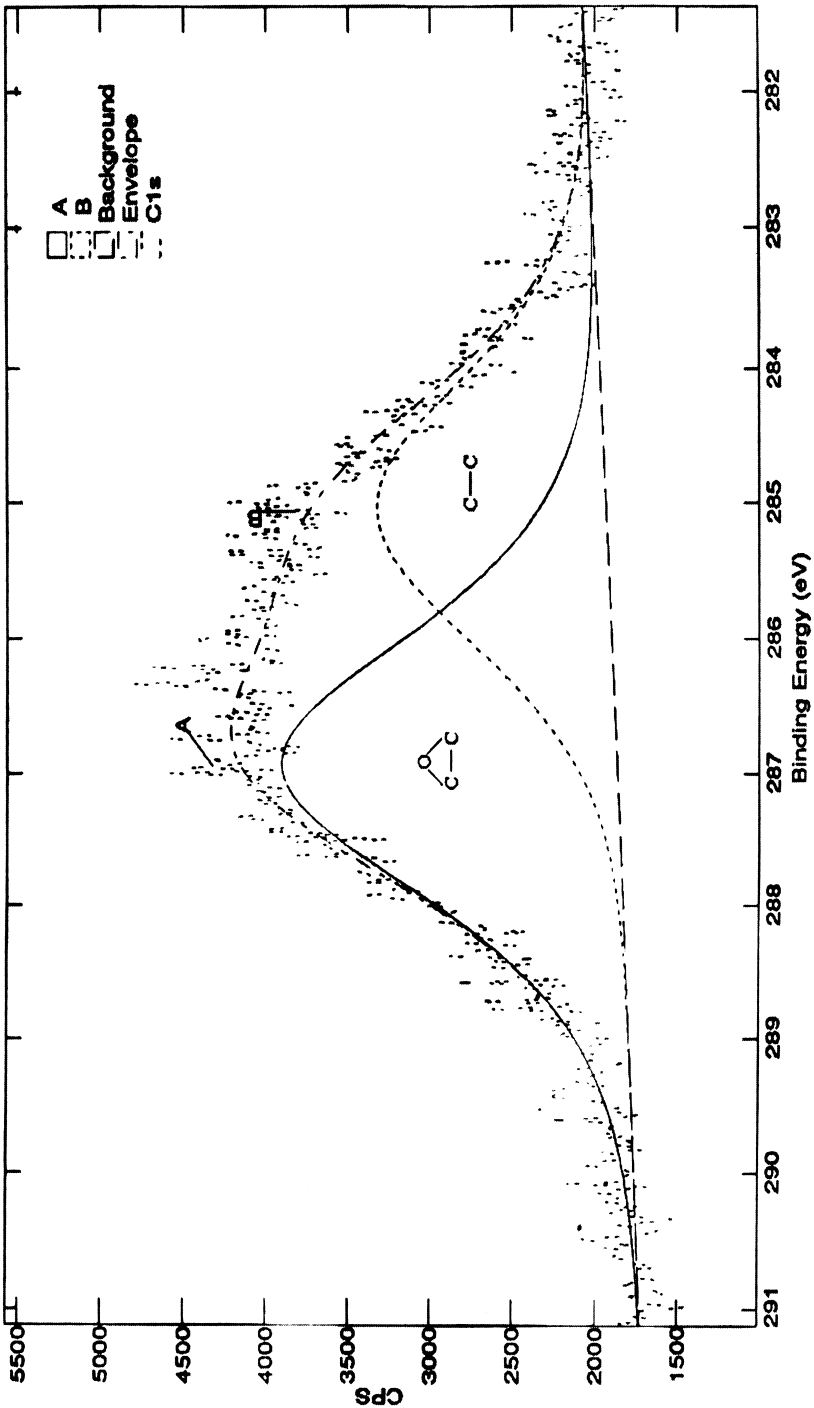

Figure 1. The XPS (C1s) spectrum of γ-glycidoxypropyl trimethoxysilane silica.

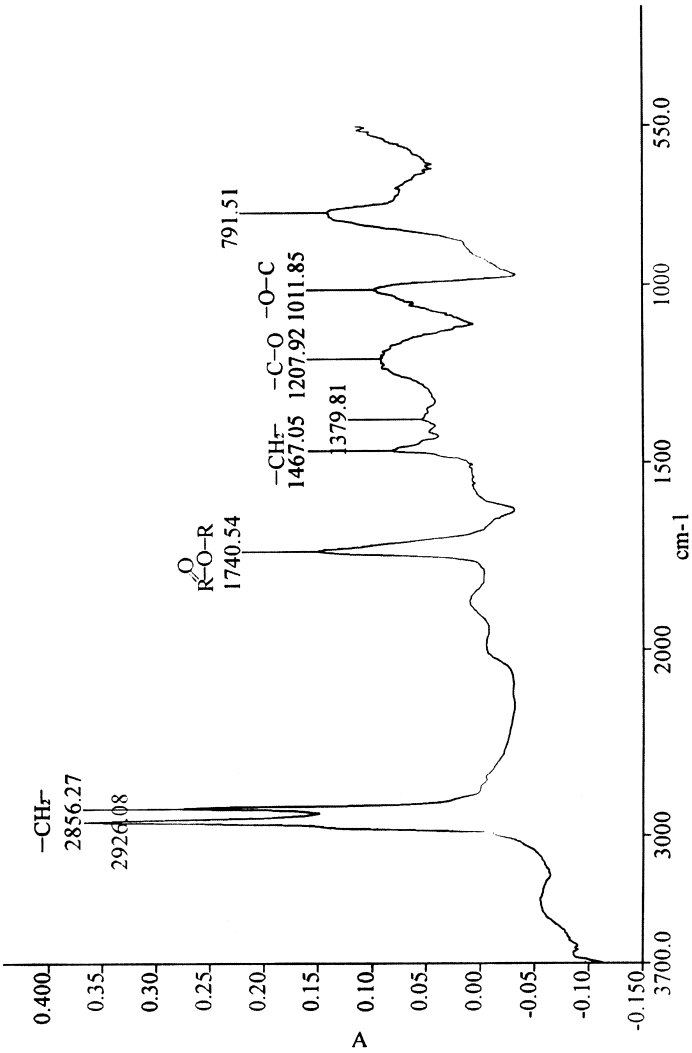

Figure 2. The FTIR spectrum of the RP packing.

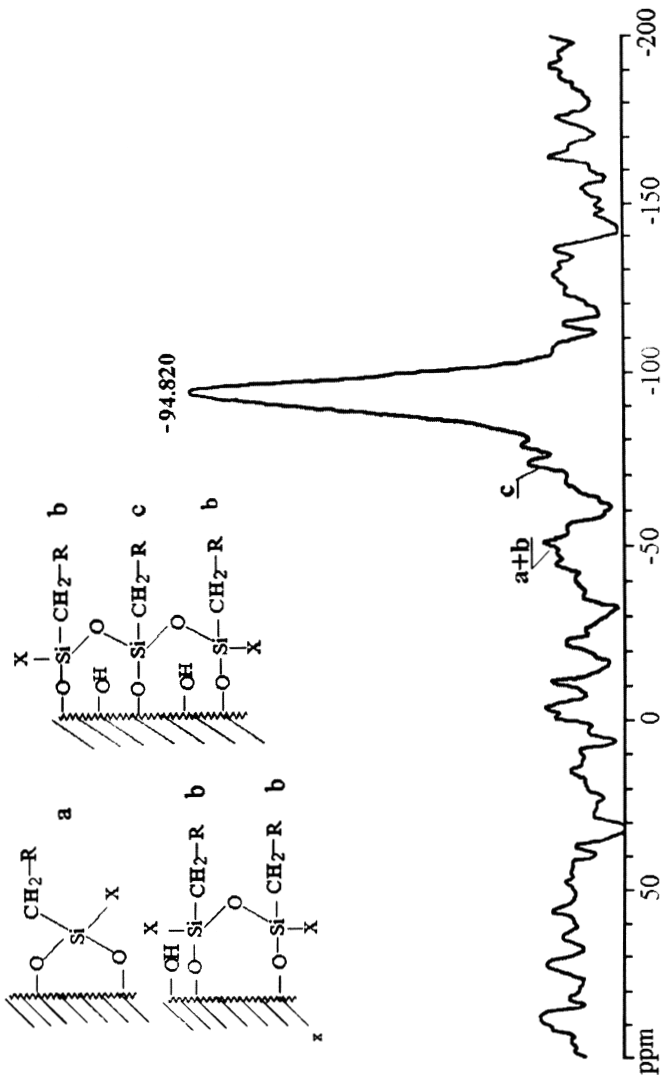

Figure 3. ^{29}Si CP-MAS NMR spectrum of the final reaction product RAS.

In the ^{29}Si CP-MAS NMR spectrum of the final product, resonance at -94.820 ppm can be attributed to tetracoordinated framework silicon; resonance around -55 and –70 ppm can be assigned to structural a, b, and c (shown in Figure 3). Further information about the alkyl chain after coupling at the surface is obtained from the ^{13}C CP-MAS NMR spectrum of the final product—RAS (figure 4). Owing to the existence of an absorption around 50 ppm, we conclude that the silica derivative contains few methoxy groups. Other information on the final product was shown in Figure 4. The peak positions in the ^{29}Si and ^{13}C spectrum are similar to those obtained for bonding similar species to silica surfaces.[10,11]

It can be concluded, that the proposed novel process for the preparation of RAS is successful from the results of elemental analysis, XPS, and solid-state ^{29}Si and ^{13}C CP-MAS NMR.

Chromatographic Evaluation

Th packing was packed into a stainless-steel column (ϕ4.0 × 50). Benzene, naphthelene, and phenanthrene were used to evaluate the RP properties of the packing, and some proteins were used to evaluate the restricted-access properties. The chromatograms on this column were shown as Figure 5 and Figure 6.

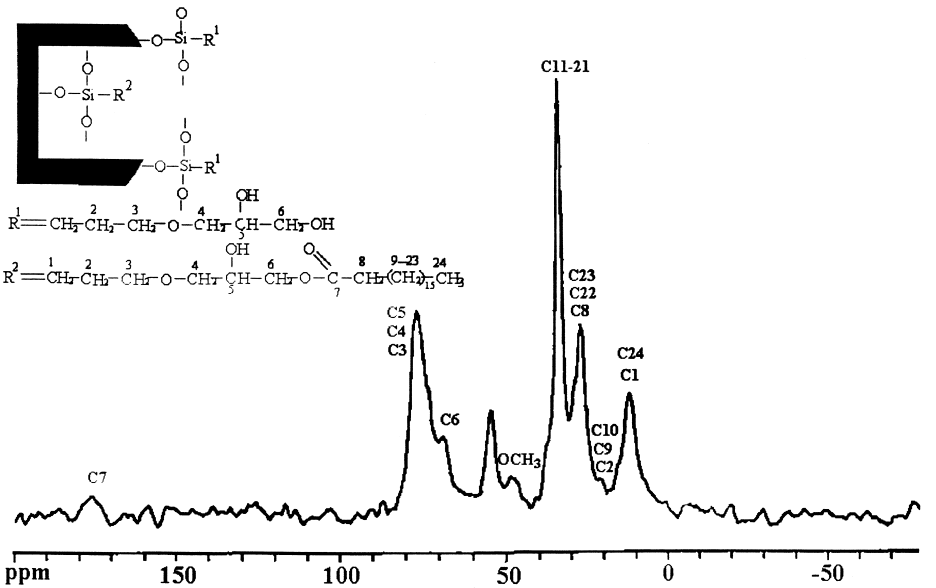

Figure 4. ^{13}C CP-MAS NMR spectrum of the final reaction product RAS.

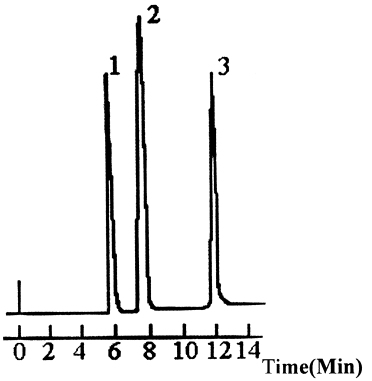

Figure 5. The chromatograms of some solutes with the RAS column described above. Column ϕ4 x 50; detection: λ254 nm, 0.1 aufs; eluent: 75% AcCN aqueous solution, 0.5 mL/min. (1) benzene; (2) naphthene; (3) phenanthrene.

In fact, small molecules in protein matrices cannot be eluted out in the condition that proteins are separated, which was confirmed through our experiment. As can be seen from Figure 5, the column performance was 64800 plates/m; the separation order of benzene, naphthelene, and phenanthrene indicated the RP-HPLC behavior of the packing.

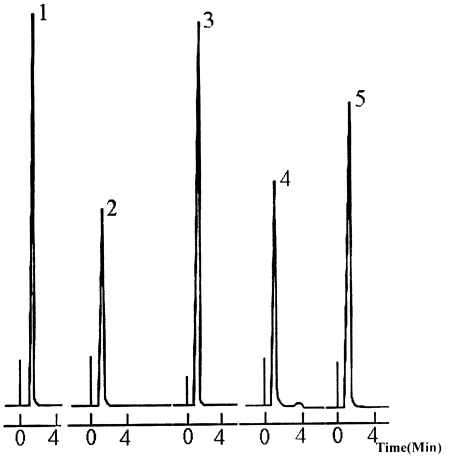

Figure 6. The chromatograms of (1) IgG; (2) hemoglobin; (3) BSA; (4) HAS; (5) Myoglobin. Eluent: 0-5% AcCN aqueous solution, 0.5 mL/min; detection: λ280 nm, 0.1 aufs.

As can be seen from Figure 6, the proteins with molecular weight from 18800 (Myoglobin) to 138000 (IgG) were separated with the same elution time when aqueous solution was used as mobile phase, which confirmed the hydrophilicity of the outer surface of the packing. With BSA, IgG, and HSA as test solutes, which were eluted through a blank column and RAS column, respectively, the protein recoveries were 97.4%, 98.4%, and 96.7%, respectively, according to their peak areas. Figure 5 and Figure 6 can demonstrate the restricted-access behavior of the prepared packing, and the successfulness of the proposed procedure for the preparation of RAS.

CONCLUSIONS

Elemental analysis, XPS, FT-IR, solid-state ^{13}C, and ^{29}Si CP-MAS NMR, and chromatographic tests, confirm the bonding of the packing and RAS behavior. As described above, the proposed method for preparation of Alkyl-Diol Silica is low toxic, economical, and simple.

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