Title

Combined paper-based substrates for electrochemical detection of copper ions in serum

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Abstract

The electroanalytical field has exploited great advantages in using paper-based substrates, even if the word “paper” might be general [1]. In fact, the mainly adopted paper-based substrates are often chromatographic and office ones. They are characterized by main features (and drawbacks): chromatographic paper is well-established for storing reagents/treating samples but the sensitivity compared to traditional screen-printed is lower (due to porosity), while office paper represents a sustainable alternative to plastic (with similar sensitivity) but its porosity is not enough to load reagents. To overcome the limitations that might arise due to the adoption of a type of individual paper-based substrate, herein we describe for the first time, the development of a 2D merged paper-based devices for electrochemical copper ions detection in serum. In this work we report a novel configuration to produce an integrated all-in-one electrochemical device, in which no additional working media has to be added by the end user and the sensitivity can be tuned by rapid pre-concentration on porous paper, with the advantage of making the platform adaptable to real matrix scenario. The novel architecture has been obtained by combining office paper to screen-print a sustainable and robust electrochemical strip, the printed electrochemical strips and chromatographic one to 1) store the reagents, 2) collect real sample and 3) pre-concentrate the analyte of interest. The novel sensing platform has allowed to obtain a detection limit for copper ions down to 5 ppb in all the solutions that have been interrogated, namely standard solution and serum, and a repeatability of ca. 10% has been obtained.

INTRODUCTION

Copper (Cu) is as an essential metal playing a crucial role in various biochemical reactions as a co-factor of many metalloenzymes and in physiological regulations. Although the majority of copper ions are tightly bound to serum proteins, i.e. caeruloplasmin, albumin, transcuprein. Even if the protein-free copper level is lower than total serum copper concentration [2], it has been highlighted that an excess of unbound copper ions could be related to tissue injury due to pro-oxidant effects and depletion of antioxidant reserves [3]. Healthy people display ca. 50 ppb (8 µM) of free copper ions in serum [4], and significant changes in serum could reflect pathological disorders like neurodegenerative, oxidative stress-related diseases and diseases related to abnormal caeruloplasmin levels, e.g., Wilson's disease (WD) and Menke's syndrome [5]. In WD, a gene mutation leads to an accumulation of copper in the liver, brain, eyes, kidneys, while in Menkes Disease, a gene mutation lead to failure of copper efflux from intestine, resulting in a low blood absorption [6]. In addition to this, free copper ions are involved in the development of some chronic degenerative diseases whose pathophysiology is largely attributed to oxidative stress, e.g. Alzheimer disease [7,8].

The determination of copper in its unbound form is of particular interest because of its high toxicity: from an analytical point of view, copper ions (and metals in general) are detected through atomic absorption spectrometry (AAS) and inductively coupled plasma-mass spectrometry (ICP-MS) [9,10]. Although these techniques display the advantage of sensibility and selectivity, they are strictly related to skilled personnel, sophisticated instruments and time-consuming procedures [11]. Depending on these experimental limitations, it is important develop easy-to-use and portable methods for decentralizing analyses. In this context, the development of portable analytical devices represents a hot topic in the analytical chemistry because it allows developing miniaturized, sustainable and low-cost devices, to be exploited for on-site measurements. In this direction, de-centralized systems have demonstrated a great potential in recent years and thus have arisen as proposed analytical tools for effective monitoring in most fields, from clinical to environmental through agri-food one. In particular, paper-based analytical devices have been highlighted as excellent and versatile substrates for developing point of care tests (POCT) [12]. Due to its valuable features such as porosity, sustainability, abundance, affordability, lightness and biodegradability, paper has been combined with colorimetric and electrochemical sensors for metals determination. In particular, different approaches have been reported for colorimetric copper sensing in diverse matrices with the use of paper-based devices: the group of Dungchai [13] developed a silver nanoparticle-modified paper-based tool for the visualization of copper ions down to micromolar in pond and tap water; another approach has exploited the combination of microspheres to absorb copper ions, metal ion indicator as the detector, SPE column and waxed-paper to determine copper in spring waters [14]; copper ions have been also colorimetrically visualized down to 15 mM with the use of ZnO@ZnS core-shell nanoparticles onto a paper-based platform [15]. Colorimetric approaches offer a valuable alternative to traditional analytical methods; however these novel approaches need to be developed together with ad-hoc settings for avoiding interference from light exposure [16]. In addition to this, an effect that should be taken into account is the possibility to analyze colored matrices that are among the most limiting factors for colorimetric analyses. An alternative in sensing and biosensing is represented by the combination between paper-based and electrochemistry, which has enforced the ASSURED criteria that has been established by the WHO [17]. The advantage of electrochemical detection over the colorimetric ones is mainly represented by the absence of interference due to the color of the matrix, especially for the biological ones. Bagheri reported a paper-based Au-decorated platform on chromatographic paper for detection of copper ions in biological fluids [18], another approach has exploited the use of gold screen printed electrode for the simultaneous determination of copper, mercury and lead in the Amazon river [19].

Even if the adoption of paper-based combined with electrochemical determination has been reported so far, the aim of the proposed work is to use a common paper, i.e. office paper, that is more robust than chromatographic paper used in the reported examples. In addition, it should be noted that the use of paper-based devices is usually limited to the adoption of this support to replace plastic-based. In this work, we took advantage, for the first time, of a 2D mixed paper-based devices for copper ions detection in serum samples. The sensitive detection of copper ions has been achieved by employing office paper to realize the printed electrochemical strips and filter paper to 1) store the reagents, 2) collect real sample and 3) pre-concentrate the analyte of interest. In this work we report a novel configuration to produce an integrated all-in-one electrochemical device, in which no additional working media has to be added by the end user and the sensitivity can be tuned by rapid pre-concentration on porous paper.

EXPERIMENTAL SECTION

Reagents and Equipment. Hydrochloric acid, acid solution (nitric acid, sulfuric acid and Trifluoroacetic acid) and serum samples were purchased from Sigma-Aldrich. Copper, lead and cadmium standard solutions (1000 mg/L) were purchased from Fluka (Germany). Nickel (II) nitrate hexahydrate and Iron (III) chloride hexahydrate were purchased by Sigma-Aldrich. Subsequent dilutions were obtained using HCl 0.1 M. All of the solutions were prepared in double distilled water. All the electrochemical measurements (linear sweep voltammetry, square wave voltammetry and differential pulse voltammetry) will be carried out using portable and battery-powered instrumentation, EmStat Blue (PalmSens, The Netherlands), connected to a laptop. Currents can be recorded and displayed on a laptop by using a dedicated app, e.g., PStouch, which can be used with all PalmSens and EmStat potentiostat.

Fabrication of paper-based platform. The homemade production of paper-based screen-printed electrodes (SPEs), starts with the choice of the paper used as a substrate, office paper (Copy 2, 80 g/m^2, Fabriano, Italy). Regarding to paper-based substrates, its porosity can represent a source of electrical noise due to diffusion of aqueous samples to connector: to avoid this, testing area is surrounded with a hydrophobic layer, melted wax, that prevents liquid flow toward the undesired portion of the strip. This is obtained through wax-printing. Firstly, the wax pattern is drawn using a drawing software (Adobe Illustrator). Then, a wax ink layer is opportunely waxed with a solid-ink printer, ColorQube 8580 from Xerox (USA). Successively, wax printed office paper was thermally cured at 100◦C for 2 min. High temperature curing (>100°C) allows wax to melt forming a hydrophobic barrier. Then, the three-electrode were manually screen-printed using a squeegee and two masks, one for the Ag/AgCl ink (Electrodag477 SS, Acheson, Italy), that was used to print the connections and the reference electrode, and one for the carbon ink (Electrodag 421, Acheson, Italy), that was used to print the working and counter electrodes. Thermally curing, 60°C for 15 min, is necessary to make the printed ink stable for electrochemical measurements [1].

AuNPs synthesis. The AuNPs synthesis started with the cleaning of all the glassware and magnetic stir bar used with aqua regia (HCl/HNO_3 3:1, v/v), rinsed in ultrapure water and then cleaned with piranha solution (H_2SO_4/H_2O_2 7:3, v/v) and rinsed again with ultrapure water before the use. Each procedure should be repeated for two times. Then, we added 9mL of double-distilled water and 1mL of the 10 mg/mL $HAuCl_4$ solution into the flask placed on the magnetic plate. Then we added 2 mL of the 10 mg/mL trisodium citrate dihydrate solution and finally, 500 mL of the 0.8 mg/mL $NaBH_4$ solution, added drop by drop. The reaction was left under stirring overnight at room temperature [20].

Cu2+ measurements. The electroanalytical technique adopted to measure Cu (II) was the Linear Sweep Voltammetry (LSV) using a AuNPs modified paper-based SPE. In particular, 0.1 M HCl was used as working solution and the method parameters have been optimized as follows: a deposition potential of -0.4 V was applied for 300 s, in a range potential from 0.1 V to 0.6 V, with a E step of 0.01 V and a scan rate of 0.5 V/s.

All-in-one configuration. In order to obtain an integrated paper-based device, the office paper-based electrode has been coupled with a waxed filter paper. The filter paper has been designed and printed with the solid ink printer, in order to obtain a multi-task paper capable to 1) store reagents (hydrochloric acid), 2) treat real matrix (serum) and 3) pre-concentrate copper ions. The scheme of the device is reported in the following Figure 1.

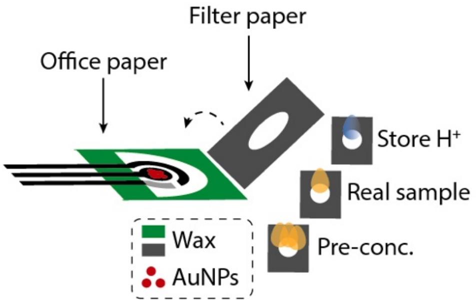

Figure 1. Schematic representation of the combination between office paper and chromatographic paper to obtain a synergic platform.

RESULTS AND DISCUSSION

Optimization of platform. The first study has been focused on the selection of the substrate for printing the conductive strips. The choice of the substrate should reflect the final application of the analytical device: plastic-based would be more suitable for application where flexibility and resistance are required, perhaps paper-based ones could represent a good compromise between sustainability and sensitivity. For this occasion, three substrates have been interrogated, namely plastic, office-paper and filter-paper. As shown in Figure 2A, same electrode configuration has been printed on the three substrates, 2 microliters of AuNP-dispersion have been drop-casted, and the metals determination has been performed on 200 ppb copper ions.

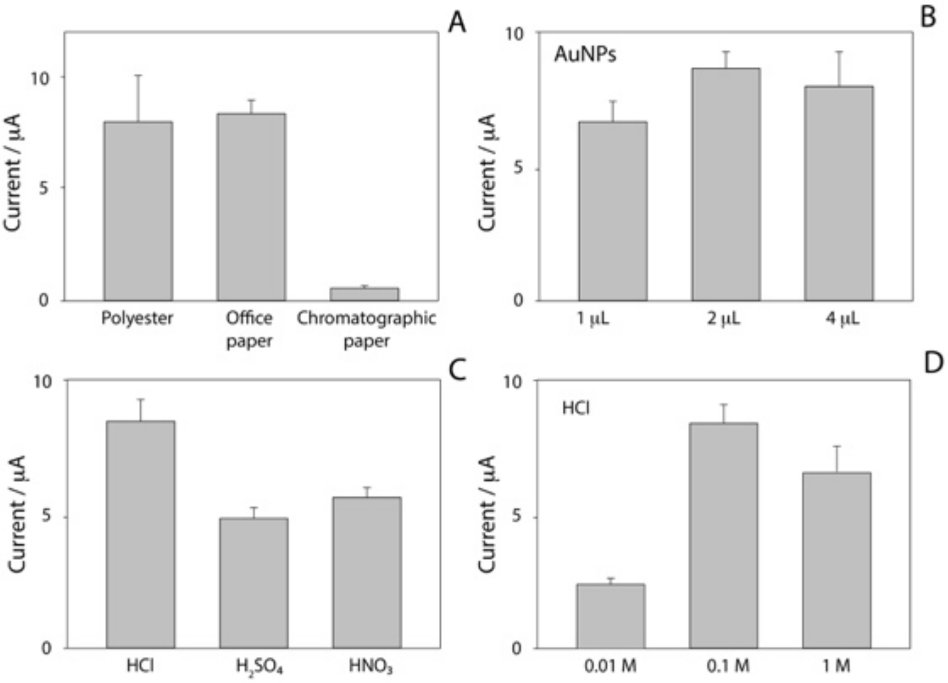

Figure 2. A) Choice of the substrate (polyester, office paper, chromatographic paper), B) evaluation of the amount of the AuNPs amount to be drop casted (1, 2, 4 µL), C) optimization of the acid used to conduct stripping analysis (hydrochloric acid, sulfuric acid, nitric acid), D) evaluation of the concentration of hydrochloric acid (0.01, 0.1 and 1 M).

As shown in Figure 2A, the sensitivity obtained with office paper and polyester is roughly the same, while the sensitivity obtained at the porous filter paper-based electrodes is low. It is attributable to the fact that with filter paper-based electrodes, the electrochemical cell is represented by the paper itself and two phenomena need to be considered: 1) AuNPs are drop casted on top of the working electrode and they are not in contact with the working solution, and 2) analyte should diffuse within paper and it is reflected with a lower resulting signal [21]. Regarding to polyester-based and office paper-based electrodes, because of the poor porosity of office paper (within the experimental condition, i.e. time, volume) and the same working electrode area that has been screen-printed, the sensitivity resulted similar within the experimental errors. Due to the possibility of developing a more "green" platform, office paper has been chosen for further studies. Consequently, the amount of AuNPs has been also evaluated as shown in Figure 2B. The amount of 2 µL has been chosen as the optimal, considering the responses obtained by modifying the electrodes with 1 and 4 µL. This could be ascribable to the fact that higher concentration of AuNPs might lead to a thick surface with the effect of limiting copper ions deposition. In addition to this, 2 µL represent the optimal compromise between sensitivity and amount of reagents. AuNPs have been used also in combination with carbon black nanoparticles, but in this case, it did not represent any sensitivity improvement. Another interrogated variable was the type and concentration of acid to be used as supporting electrolyte for copper quantification. As displayed in Figures 2C and 2D the best results have been obtained with the use of 0.1 M hydrochloric acid. This is in accordance with other works that have been reported in literature [19,22], and a it might be due to the different counter ions that can affect the availability of copper ions within the working solution.

Electrochemical parameters optimization. After having optimized the parameters related to the printing substrates, AuNPs and acidity of the working solution, the optimization of the electrochemical parameters has been taken into account. According to the electrochemical detection technique that has been used, i.e. Linear Sweep – Anodic Stripping Voltammetry, the three most relevant factors have been optimized: deposition potential, deposition time and scan rate of the stripping wave, as shown in Figure 3. All the experiments have been carried out in presence of 200 ppb of copper ions in 0.1 M hydrochloric acid.

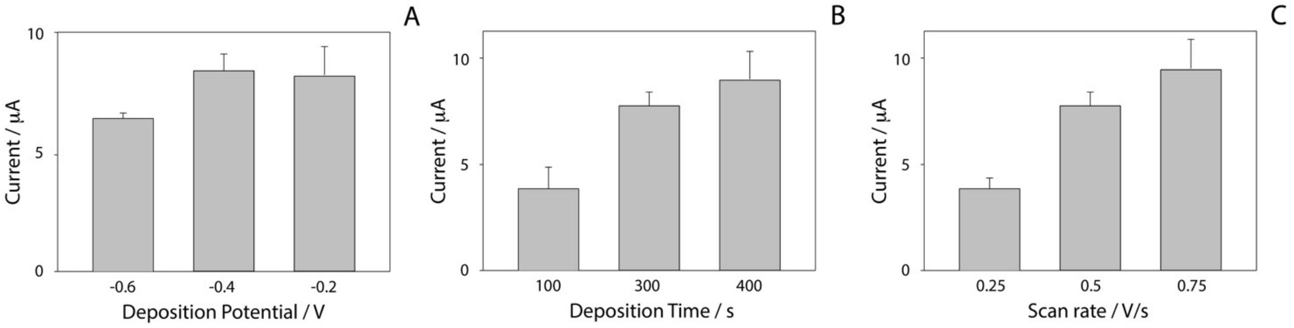

Figure 3. A) Optimization of the deposition potential (-0.6, -0.4 and -0.2 V), B) Choice of the deposition time between 100 and 400 s, 3) scan rate of the linear sweep (0.25, 0.5 and 0.75 V/s).

The deposition potential, required to reduce Cu2+ to Cu0, has been studied from −0.6 to -0.2 V, as reported in Fig. 5A. Thus, -0.4 V has been chosen as the optimal deposition potential because of its higher sensitivity and repeatability with respect to other potentials that have been taken into account. Then, the deposition time has been evaluated from 100 to 400 s. This time is necessary to allow copper ions to reach the working electrode surface, and in absence of stirring procedures, only diffusion of copper ions occurs. A deposition time of 300 s has been chosen even if the sensitivity appeared to be lower if compared with a 400 s of deposition: this is due to the fact that waiting 100 s more did not lead to proportional advantage in terms of signal intensity, thus 300 s represented the optimal compromise between sensitivity and analysis time. Finally, the scan rate of the anodic stripping has been considered, and in the range comprised between 0.25 and 0.75 V/s. 0.5 V/s has represented the optimal compromise between signal intensity and repeatability of the measurements, as shown in Figure 3C.

Analytical performance of office paper towards copper ions. Once both the platform and electrochemical parameters have been optimized, the office paper-based sensor has been tested for determination of copper ions within the 0-300 ppb range in standard solution as reported in Figure 4.

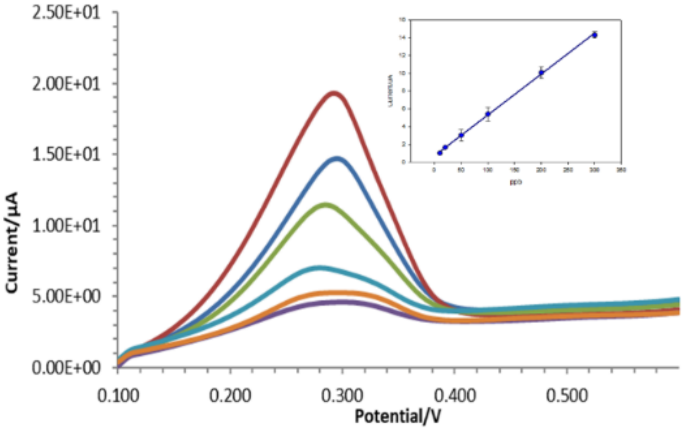

Figure 4. Voltammograms recorded performing LS-ASV from 0 to 300 ppb of Cu^{2+} in 0.1 M HCl (standard solution) using office paper-based platform. Inset: calibration curve in a linear range up to 300 ppb. E dep = -0.4 V; t dep = 300s; E begin = 0.1 V; E end= 0.6 V, E step = 0.01 V; scan rate = 0.5 V/s.

As shown in Figure 4, a linear correlation has been obtained between the height of the current peaks and the concentration of copper ions. The relationship is described by the following equation, y = 0,046x + 0,730 (where y represents the current difference between copper and the blank, and x represents the copper level expressed in ppb). The detection limit, calculated as 3σB/slope, has been calculated equal to 4.7 ppb and the limit of quantification has resulted equal to 14 ppb, with a repeatability of 11% (calculated on three replicates). Successively, the all-in-one approach has been evaluated. Briefly, a filter paper-based substrate has been used to load the hydrochloric acid and also to pre-concentrate the sample by dropping on the wax-patterned filter paper increasing amount of sample to be measured.

Serum measurements. In order to demonstrate the application of the platform for the detection of copper ions in serum, the selectivity has been investigated. In particular, the selectivity of the paper-based platform has been evaluated in the presence of interfering species that can affect the measurements, as shown in Figure 5.

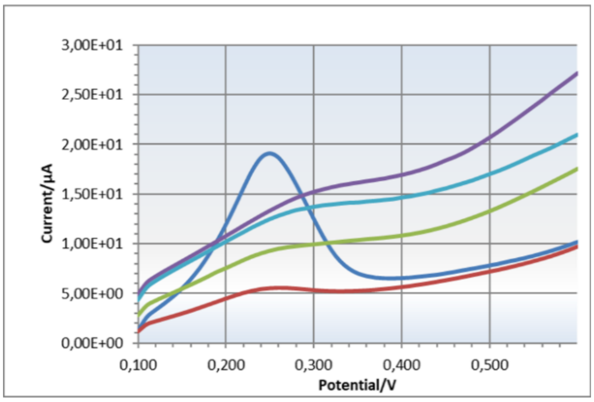

Figure 5. Selectivity studies in presence of copper (blue), nickel (green), cadmium (violet), iron (red) and lead (cyan) at the ppb level in 0.1 M hydrochloric solution using the optimized parameters (reported in caption of figure 4).

As shown in Figure 5, none of the tested interferents has produced a significant signal that can affect the measurements of copper ions. The analytical efficacy of the developed electrochemical sensor for the determination of copper ions has been also demonstrated by applying it to the determination of copper ions in commercial serum. The first study has involved the evaluation of the matrix effect (Figure 6A). It should be noted that the presence of this complex matrix has produced a decrease of the sensitivity due to the presence of species like proteins that can foul the electrode surface by adsorption phenomena [21].

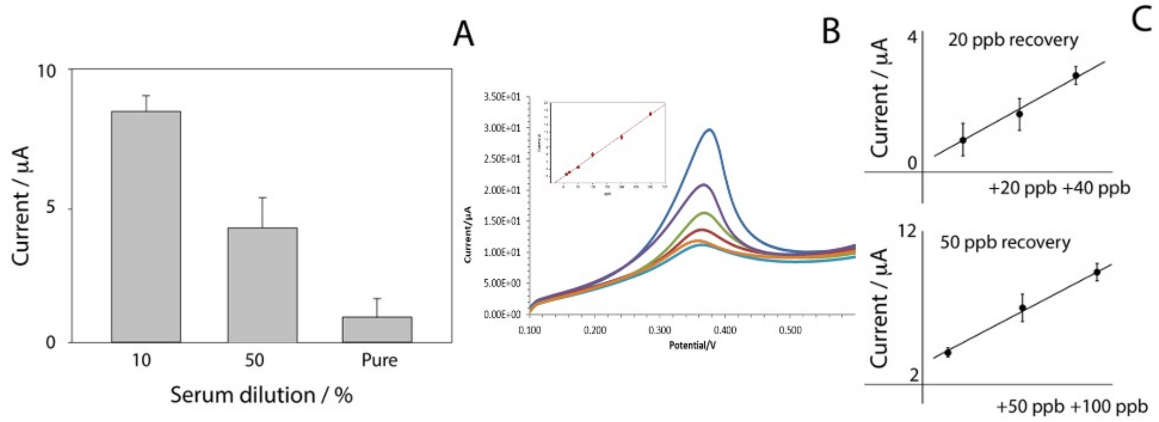

Figure 6. A) Evaluation of the effect of the matrix with the use of hydrochloric acid 0.1 M (10%, 50%, untreated), B) Calibration curve in commercial serum diluted 10% (same parameters as Figure 4), C) recovery studies in presence of 20 and 50 ppb spiked serum samples.

Commercial serum has been investigated and 10%-dilution has produced the highest sensitivity. Consequently, serum sample has been spiked with copper ions level up to 300 ppb. As shown in Figure 6B, a good linearity has been obtained, in good agreement with the results that have been obtained in standard solution and the detection limit in serum has been calculated equal to 4.5 ppb. It should be noted that same sensitivity of the platform has been obtained by sampling serum directly on the filter paper, used both for reagents loading and for sample pre-concentration. In addition, a pre-concentration step on filter waxed-paper, performed with three consecutive additions of serum samples containing 20 and 50 ppb copper ions, highlighted similar results compared with the direct detection of 60 and 150 ppb solutions. In order to evaluate the accuracy of the method, recovery studies have been carried out at two different levels of copper ions, namely 20 and 50 ppb, obtaining 81 and 84% recovery respectively (Figure 6C).

CONCLUSION

This work demonstrates the development of a simple and sustainable method to detect copper ions in a biological matrix like serum. Screen-printed electrodes were easily produced onto office paper and effectively customized with gold nanoparticles by a drop casting procedure. The detection limit of the platform has reached less than 5 ppb in both standard and serum solution. In addition, it should be noted that an ulterior filter paper-based platform has been used to pre-load the reagents and pre-concentrate real samples, with the advantage of making the platform adaptable to real matrix scenario. We demonstrate that filter paper can act in combination with office paper to avoid tasks for end user, i.e. acidification. The next challenge will be focused on the development of a customized 3D-printed case that will make the platform integrated and ready to market.

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