Superhydrophobic surface-based magnetic electrochemical immunoassay for detection of Schistosoma japonicum antibodies

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1. Introduction

Schistosomiasis is caused by Schistosoma worms (e.g., Schistosoma japonicum, Sj) and occurs primarily in Asia, Africa, and Latin America (Li et al., 1991). Resurgence of this formidable parasitic disease has led to intensified interest in developing more efficient diagnostic tests. Some conventional immunoassay methods have been adapted to clinical analyses (Li et al., 1991), including radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), electrophoretic immunoassay, etc. Unfortunately, most methods require time-consuming procedures, potentially dangerous materials, and expensive instruments. In addition, these methods have been mainly used for qualitative or semiquantitative analysis for Sj antibodies (SjAb). In recent years, some efforts have been alternatively dedicated to the quantitative detection of SjAb by using quartz crystal microbalance (Wang et al., 2006), electrochemical (Chu et al., 2005; Liu et al., 2001), fluorescent (Che et al., 2008), or chemiluminescent (Qing and Chu, 2008) immunoassays. Electrochemical immunoassays have evolved dramatically over the last decade and benefit from high sensitivity, inexpensive instrumentation, and simplification of operation (Fu et al., 2010; Guo and Dong, 2009; Kerman et al., 2008; Tang et al., 2010). Moreover, they are easy to miniaturized to develop portable analytical devices for meeting the portability requirements of decentralized point-of-care testing, epidemic screening of infection diseases, or field-based detection of biological warfare agents or field detection of bioagents (Liu et al., 2004; Zhang et al., 2009). Liu and colleagues prepared a paraffin–graphite–Sj antigen (SjAg) bicomposite to develop a renewable amperometric immunosensor for a competitive assay of SjAb with a detection limit of 360 ng ml −1 (Liu et al., 2001). More recently, Chu and co-workers reported a noncompetitive sandwich immunoassay for stripping voltammetric detection of SjAb with the use of silver-enhanced Au nanoparticle (AuNP) label amplification (Chu et al., 2005). Although a lower detection limit (3 ng ml −1 ) of SjAb was achieved, this approach carried out on the polystyrene microwells might still suffer from some intrinsic disadvantages such as requirement of large volumes of reagents or samples (e.g., 200 μl for a single biorecognition step), low reaction kinetics, and tedious assay time (e.g., 60–70 min for a single biorecognition step).

In recent years, magnetic electrochemical bioassays with functional nanomaterial labels have attracted increasing interests for detection of biological analytes (Kawde and Wang, 2004; Wang et al., 2008; Wang and Liu, 2003; Wang et al., 2001). Compared with heterogeneous bioassays performed on planar platforms such as microwells, magnetic particle-based suspension bioassays show

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faster reaction kinetics in solution due to the radial diffusion of analytes or probes, leading to shorter assay time. Additionally, highly efficient magnetic separation could minimize nonspecific adsorptions (Liu et al., 2004). Wang et al. combined magnetic separation with silver-enhanced AuNP label (Wang et al., 2001) or gold-enhanced multi-AuNP label (Kawde and Wang, 2004) amplification for rapid, highly sensitive stripping voltammetric detection of DNAs. Nevertheless, it currently still remains a great challenge to reduce the total volume of biomagnetic particle probes and samples or reagents used for a single biorecognition step.

In this paper, we introduce a superhydrophobic surface-based analytical platform (SSAP) that addresses many of these limitations. Lotus leaf-inspired superhydrophobic surfaces, with a water contact angle (CA) larger than 150° as well as a water rolling angle (RA) lower than 10°, have attracted extensive research attention over the last few years (Erbil et al., 2003; Han et al., 2010; Lu et al., 2004; Park et al., 2010; Sun et al., 2005). Due to their unusual water-repellent property, superhydrophobic surfaces can find wide fundamental and practical applications, such as self-cleaning surfaces, microfluidic devices, separation equipments, and non-loss liquid transportation. However, few superhydrophobic surface-based immunoassays have been reported so far. Recently, we proposed a simple phase-separation method for creating superhydrophobic polycarbonate (PC) coatings onto various material substrates (Zhang et al., 2008). They additionally retain superhydrophobicity in whole pH range and have long-term mechanic stability. We expect that they will also be useful in bioapplications such as immunoassays. Herein, such PC coatings are modified onto the inner wall surfaces of commercially available polymer test tubes to fabricate the SSAP for developing copper-enhanced AuNP label magnetic electrochemical immunoassays for the detection of SjAb. This new SSAP-based SjAb immunoassay method is overall proved with rapid, sensitive, selective, and small-volume analysis features in examining real serum samples.

2. Experimental

2.1. Reagents

Ascorbic acid, CuSO₄·5H₂O, HNO₃, K₂CO₃, HCl, and poly-(ethylene glycol) (PEG, M₉ 6000) were the products of Tiantai Fine Chemistry and Engineering Company (Tiantai, China). H₂AuCl₄·H₂O, citrate sodium, and glutaraldehyde were purchased from Sigma–Aldrich. Amine-coated superparamagnetic particles (MPs, 0.5–1 μm diameter, 5 mg ml⁻¹) were from Tianjin BaseLine ChromTech Research Centre (Tianjin, China). Phosphate-buffered saline (PBS) solution (pH 7) containing 0.9% NaCl was prepared with 0.067 M Na₂HPO₄ and 0.067 M KH₂PO₄. The copper enhancer solution was a 1:1 mixture of 0.1 M ascorbic acid and 0.18 M CuSO₄·5H₂O (Mao et al., 2007). Acetate buffer (pH 4.5) supporting electrolyte solution was prepared with 0.2 M acetic acid and sodium acetate. All other reagents were of analytical reagent grade. Deionized water (specific resistivity > 18 MΩ cm) used throughout experiments was obtained from an ultrapure water system that was provided by Barnstead/Thermolyne Corp. (Dubuque, USA).

SjAb (4.2 mg ml⁻¹, M₉ 32,000) was obtained from soluble homogenate of adult Sj worms through an elaborated purification procedure as described in the literature (Wang et al., 1995). Infected rabbit serum (IRS) and normal rabbit serum (NRS) samples were kindly provided by the Institute of Schistosomiasis, Xiangya School of Medicine, Central South University (Hunan, China). The actual concentrations of Sj antibodies (SjAb) in IRS samples with various infected degrees were determined by the ELISA method. The IRS sample with 2 mg ml⁻¹ SjAb was diluted by NRS to obtain desired analyte concentrations used as the calibration standard. Normal human serum (NHS) samples were kindly provided by the campus hospital, Hunan University (Hunan, China). The goat anti-rabbit IgG antibodies and bovine serum albumin (BSA) were obtained from Hefei Bomei Biotech. Co. Ltd. (Hefei, China).

2.2. Apparatus

The square-wave stripping voltammetric (SWSV) measurements were performed on a LK 2005A electrochemical analyzer provided by Tianjin Lanlike Chemical and Electronic High Technology Co. Ltd. (Tianjin, China), using a three-electrode system consisting of a glass carbon working electrode (2 mm diameter), a saturated calomel reference electrode (SCE), and a platinum wire counter electrode. The measurements of water CAs and RAs for the prepared superhydrophobic PC coating were conducted on a Powerecah JC 2000C goniometer (Shanghai Zhongchen Digital Technology Co. Ltd., China) with water droplets of 4 μl. The water RAs were defined as described elsewhere (Han et al., 2010), typically the substrate coated with the PC coating was inclined gradually from 0° to higher angles till the water droplet would roll down the coating surface. The surface morphology of the PC coating was characterized by a scanning electron microscope (SEM, JSM-5600LV, JEOL, Japan) at an acceleration voltage of 20 kV.

2.3. Preparation of SSAP

The SSAP was prepared by modifying the inner wall surfaces of 10-ml polypropylene tubes having specific dimensions of 1.5 cm (inner diameter) by 1.5 cm (height) with superhydrophobic PC coatings according to the method reported in our previous work (Zhang et al., 2008). Briefly, commercial bisphenol A-type PC particles were dissolved into dichloromethane solution with a final concentration of 10 wt%. The special polymer tube was filled with the PC solution for 5 min, followed by a fast evaporation of dichloromethane at room temperature. The resultant tube was then immersed into 1,4-dimethylbenzene for 1 min. The tube modified with PC coating was subsequently left for 15 min to evaporate these organic agents till a white surface appeared. Twenty four PC-coated tubes were prepared individually in this way and further integrated together using adhesive tape to construct a 24-well SSAP.

2.4. Preparation of MP–SjAg conjugates

The magnetic particle-SiAg (MP–SjAg) conjugates were prepared as follows. Typically, 1 ml of MP suspension (2 mg ml⁻¹) was mixed with 5 ml of glutaraldehyde (5%). The reaction mixture was rotated for 3 h at room temperature. Subsequently, magnetic separation was conducted to remove the un-reacted glutaraldehyde. After being washed four times with PBS buffer (pH 7), 1 ml of 4.2 mg ml⁻¹ SjAg were added to the aldehyde-activated MPs. The mixture was further rotated for 3 h at room temperature, followed by magnetic separation and two washings. Subsequently, the MP–SjAg conjugates were re-suspended in 4 ml of PBS buffer (pH 7), into which 0.5 ml of NRS and 0.5 ml of BSA (10 mg ml⁻¹) were introduced to be further rotated for 1.5 h at room temperature to block the remaining aldehyde groups that were left on the MPs. Magnetic separation and washings were conducted in turn. The resultant MP–SjAg conjugates were re-suspended again in 1 ml PBS buffer (pH 7, containing 1.5% PEG) and stored at 4°C. The as-prepared MP–SjAg conjugates remain stable with no significant change in immunoactivity for 3 months.

2.5. Preparation of AuNPs and their bioconjugates

The preparation of Au nanoparticles (AuNPs) with an average diameter of approximately 15 nm and AuNP–anti-rabbit IgG
antibody (AuNP–Ab) conjugates were accomplished according to the method described in our previous work (Zhang et al., 2009). Basically, 100 ml of 0.01% HAuCl₄ solution was heated to boil with gentle stirring. As soon as the solution was boiling, 4 ml of 1% trisodium citrate was rapidly added, and boiling was pursued for an additional 9 min. Then, the solution was cooled to room temperature and stored at 4 °C. To prepare AuNP–Ab conjugates, 0.6 ml of anti-rabbit lgG (1 mg ml⁻¹) was added into 10 ml of pH-adjusted AuNP solution (pH 9 adjusted with 0.1 M K₂CO₃), followed by periodic gentle mixing for 1 min and then keeping the solution quiescent at room temperature for 1 h. The conjugates were then centrifuged at 18,500 rpm for 20 min. The soft sediments obtained were rinsed by 10 ml of 10 mg ml⁻¹ BSA in PBS (pH 7) and collected after a second centrifugation at 16,000 rpm for another 20 min. Finally, the AuNP–Ab conjugates were re-suspended in 0.5 ml of NRS and 0.5 ml of BSA (10 mg ml⁻¹) and stored at 4 °C before use. The as-prepared AuNP–Ab conjugates might remain stable with no significant change in immunoactivity for 1 month.

2.6. Noncompetitive sandwich immunoassays

An 8-μl aliquot of MP–SjAg conjugate suspension (1 mg ml⁻¹) was added into each tube of the 24-well SSAP that was pre-washed with water. Then, 2-μl aliquots of IRS samples, with desired SjAg concentrations in PBS buffer (pH 7), were separately introduced into the tubes, and the SSAP was slightly shook on a rocking bed at a quite low running rate of 60 rpm for 20 min at room temperature. The mixtures were subsequently magnetically separated, and washed twice with PBS buffer. The magnetic sediments in each tube were re-suspended in a 10-μl aliquot of AuNP–Ab labels (1/8, v/v), and mixed for another 20 min. The resulting sandwich immunocomplex-loaded MPs in each tube were washed twice with PBS buffer and water, and then re-suspended in 10 μl of copper enhancer solution for shaking for 10 min. After a magnetic separation and a thorough washing, a 10-μl aliquot of 1 M HNO₃ was added into each tube to dissolve metal copper deposited on the surfaces of the AuNPs. After a 3-min shaking and another magnetic separation, the HNO₃ solution containing the released copper ions was transferred into an electrochemical cell containing 0.1 M acetate buffer solution (pH 4.5, containing 10 μg ml⁻¹ mercury ions). Moreover, control experiments were performed in a similar fashion but using 2-μl of NRS, NHS, and BSA instead of IRS. Bare 1.5-ml polypropylene tubes were used as the replacements of the 24-well SSAP to conduct the comparison experiments.

2.7. Electrochemical detection

SWSV measurements of the released copper ions were performed (in a stirring acetate buffer solution) with an in situ plated mercury film formed on a glassy carbon electrode by a 1-min pretreatment at 0.6 V and a 2-min accumulation at −1.4 V. The analysis parameters mainly included the applied potential range of −0.6 to 0.2 V, a step potential of 4 mV, an amplitude of 20 mV, and a frequency of 25 Hz. Note: the waste after each immunoassay should be collected in a bottle to be disposed of safely.

3. Results and discussion

3.1. SEM and wettability characterization of the SSAP

Fig. 1 shows the picture (in top view) of the 24-well SSAP made of 24 polypropylene tubes modified with white superhydrophobic PC coatings. Such a 24-well SSAP can allow 24 different immunoassay experiments to be conducted simultaneously. Larger assay throughput may be achieved by integrating more superhydrophobic tubes. As shown in the typical SEM image of the superhydrophobic PC [Supplementary material, Fig. S1], this polymer coating exhibits a coral-like surface morphology that is generated with large numbers of micrometer-scale spherulites decorated with nanometer-sized mastoids of fine structures. As lot of air is trapped into the cavities between mastoids or papillae of PC coating, water can only contact with the tips of the micrometer-scale mastoids (Zhang et al., 2008). This may essentially explain the approximately spherical aspect of the water droplet that shows a high CA of 160° [Supplementary material, Fig. S1, insert]. The RA was also investigated for this PC coating. It was found that a slight incline of the SSAP (less than 5°) would make the water drop immediately roll off the PC surface.

3.2. SSAP-based magnetic electrochemical immunoassay

Fig. 1 also schematically illustrates the analytical principle and procedures of the SSAP-based magnetic electrochemical immunoassays for the detection of SjAb in a noncompetitive sandwich format. SjAb in the sample is first captured by MP–SjAg conjugates. AuNP–Ab is then introduced to selectively recognize the captured SjAg. The resultant immunocomplex is subsequently treated by a copper enhancer solution to enlarge the bound AuNPs via the catalytic reduction of copper ions. Finally, the deposited metal copper is dissolved in an acid, followed by the SWSV measurement of the released copper ions. The yielded SWSV signals are proportional to the SjAb concentrations in the samples. In comparison with the commonly-applied silver (Chu et al., 2005) and gold (Liao and Huang, 2005) enhancer solutions used for AuNP enlargement, the copper enhancer solution originally reported by Shlyahovsky and co-workers has several advantages (Shlyahovsky et al., 2005), such as easier to prepare and preserve, more safe to use, etc. Moreover, the method chosen for measuring the released copper ions is the SWSV technique, as it combines the amplification feature of stripping voltammetry with the speed advantage of square-wave scanning (Wang, 1985).

3.3. Performance comparison between different platforms

To examine the advantages of using superhydrophobic surface, we make a performance comparison between the developed SSAP and bare polymer tubes (without the modification of superhydrophobic PC coatings) primarily used in conventional suspension bioassays. Fig. 2 represents the typical SWSV responses obtained from the electrical immunoassays performed on the two platforms for four different samples, respectively. The special experimental parameters with regard to the SjAb concentration in IRS sample and the analytical time for a single immunoresponse step are 100 ng ml⁻¹ and 20 min for the SSAP, respectively, and 200 ng ml⁻¹ and 40 min for the bare tubes. As shown in Fig. 2, for either the SSAP or the bare tubes, well-defined copper SWSV signals (peaked at −0.22 V) are observed in the presence of SjAb in IRS, and substantially smaller signals occur in the absence of analyte in NRS, NHS, or the presence of 1000-fold excess of BSA foreign proteins. These results indicate that the adopted magnetic electrochemical immunoassays with copper-enhanced AuNP labels possess good selectivity. The minimization of nonspecific adsorptions might be attributed to the synergic blocking effects of BSA and NHS that were applied for both MP–SjAg conjugates and AuNP–Ab labels (Wang et al., 2006). Another factor that may contribute to the reduced nonspecific adsorptions is the highly effective magnetic separation, i.e., removal of foreign molecules from complex sample backgrounds (Liu et al., 2004).

More importantly, substantial differences in the copper SWSV responses obtained from the analyte detection are observed between the SSAP and the bare tubes, with the former displaying more favorable signal characteristics as shown in Fig. 2. The peak
current signal of copper obtained from the SSAP is over 3 times greater than that obtained from the bare tube (131.7 μA vs 42.5 μA), though higher SjAb concentration and longer reaction time are used for the latter. The different responses reflect the changes in the wettability of the two platforms. The inner wall surface of the bare tube shows low hydrophobicity, for example, a small-volume of reactant suspension solution consisting of 8 μl MP-SjAg conjugates and 2 μl IRS solution shows a very low CA of 98° (Supplementary material, Fig. S2, up). Moreover, when the bare polymer substrate is placed upside down, the reactant solution even does not drop off (Supplementary material, Fig. S2, up), displaying a quite serious hysteresis behavior. Thus, the reactant solution would lack an effective movement and mixing when the bare tube is slightly shook on the rocking bed at a low running rate (i.e., 60 rpm). Accordingly, most MP-SjAg probes precipitate at the bottom of the reactant solution because of the force of gravity (Supplementary material, Fig. S2, up), and they are not able to re-suspend again to capture the SjAg analyte even with a prolonged shaking manipulation (Supplementary material, Fig. S2, up). This condition may also take place in the subsequent recognition of magnetic immunocomplex by AuNP-Ab labels. The resultant low antigen–antibody binding efficiency finally leads to the low copper SWSV signal.

For the SSAP, on the other hand, the high PC coating’s CA of 155° (Supplementary material, Fig. S2, down) can allow the 10 μl of reactant suspension droplet to keep an approximately spherical aspect. In addition, its low RA (less than 5°) can make the suspension droplet easily roll on the PC surface (Supplementary material, Fig. S2, down). Accordingly, though all MP-SjAg probes precipitate at the bottom of the reactant solution, they are able to re-suspend again to capture the SjAg analyte as long as the applied external force is just strong enough to make the SSAP shake or sway (Supplementary material, Fig. S2, down). Such a suspension droplet may be considered as a “self-circulating flowing reactant system” where a highly effective mixing of MP–SjAg conjugates and IRS sample could be achieved to realize high antigen–antibody binding efficiency. Similar effective recognition processes could also occur between the magnetic immunocomplex and AuNP–Ab labels. Therefore, larger copper SWSV signal is obtained on the SSAP even using a shorter assay time and an IRS sample containing lower SjAb concentration.

Note that, a smaller total volume (e.g., 5 μl) of biomagnetic particle probes and samples or reagents, which can also form a “self-circulating flowing reactant system”, is not recommended for use in a single analytical procedure as it lacks the convenience for hand-operation in practice. In addition, since no reagents or samples contaminate the superhydrophobic PC surface, the SSAP is self-cleaning and could be reused. The lifetime (mechanical stability) of the SSAP has been investigated. The water CA of a PC-coated tube was measured after one measurement was finished. It was experimentally found that after 369 measurements the reactant suspension droplet would adhere to the polymer surface with a water CA of ~141°. In other words, the SSAP can be reused for 369 measurements. It should be pointed out that the SSAP could not be used for SjAb assay again if the micro-nano-scale surface
structures of the PC coatings were intentionally destroyed by a pipette tip or other sharp objects to reduce its water-repellent ability. Thus, attention should be paid to the operations carried out in the SSAP, such as the addition or removal of reactant solution droplet.

3.4. Quantitative analysis

The main experimental factors for the SjAb immunoassays have been optimized in detail, including the used amounts of MP–SjAg conjugates and AuNP–Ab tags, reactive times for both magnetic capture and AuNP–Ab recognition of SjAg, and the copper-enhancing reaction time (Supplementary material, Fig. S3).

To investigate the quantitative analysis capabilities of the developed immunoassay, a series of serum samples with varying SjAb concentrations were assayed under optimal experimental conditions. Fig. 3 displays the resulting calibration curve between peak currents versus logarithmic concentrations of SjAb. As shown in Fig. 3, linear responses are obtained over the concentration range of 2 ng ml\(^{-1}\)–15 ng ml\(^{-1}\) SjAb. The sensitivity of this method was accordingly calculated to be \(\sim 7.3 \mu \text{A ng}^{-1} \text{ml} \), a detection limit was estimated to be \(\sim 1.3 \text{ng ml}^{-1}\), as estimated by three times of signal-to-noise ratio (S/N = 3). Moreover, five repetitive measurements were performed using 10 ng ml\(^{-1}\) and 100 ng ml\(^{-1}\) SjAb samples to estimate the precision of the analysis. The mean stripping peak currents were 63.7 \(\mu \text{A}\) and 132.9 \(\mu \text{A}\), and the relative standard deviations were 5.2% and 7.3%, respectively. Such signal variations reflect the favorable detection reproducibility of the proposed detection system.

In addition, the comparison results of the overall analytical performance between the new SjAb assay method and the recently reported ones based on quartz crystal microbalance (QCM) (Wang et al., 2006), electrochemical (Chu et al., 2005; Liu et al., 2001), fluorescent (Che et al., 2008), and chemiluminescent (Qing and Chu, 2008) techniques are summarized in Table 1. One can find from Table 1 that the developed SSAP-based magnetic electrochemical immunoassay requires lower volumes of reagent and sample and shorter assay time, and has a lower detection limit, compared with other SjAb assay methods.

3.5. Analytical applications

The developed magnetic electrical immunoassay system was applied to evaluate four IRS samples with varying degrees of Sj infection, with the results shown in Table 2. The SjAb concentration is positively correlated with the degree of Sj infection that increases with increases in number of infected Sj or days of infection. One notices that the current responses increase with the increases in the infection degree. That is, this novel SjAb immunoassay protocol can easily define the degree of Sj infection of IRS samples. To further investigate its feasibility for practical
applications, other six IRS samples were assayed, and the obtained results were compared with those obtained by the ELISA performed at the Institute of Schistosomiasis, Central South University (Fig. 4). A regression equation of \( y = 1.0631x + 2.2759 \) and a correlation coefficient of 0.9654 are obtained. The statistically significant difference estimated by the common \( F \)-test showed no significant difference \((P>0.05)\) between the results of the two methods. Moreover, sixty-seven serum samples were assayed by the novel technique, and the false negative rate and false positive rate obtained were 1.0% and 2.5%, respectively. These results suggest that the developed magnetic electrochemical immunoassay should be comparable to ELISA, thus providing an alternative tool that might be used for the determination of \( \text{SjAb} \) concentration in clinical real serum samples.

4. Conclusions

We have successfully fabricated a lotus leaf-inspired novel analytical platform, namely the SSAP for the development of a new magnetic electrochemical immunoassay of \( \text{SjAb} \) in IRS samples, based on the association of sensitive stripping voltammetry analysis with the copper-enhanced AuNP amplification. The nonspecific adsorption effects can be minimized by using a synergetic blocking agent of BSA and NRS in addition with efficient magnetic separation. Due to the superhydrophobic PC’s unusual water-repellent and self-cleaning ability, the SSAP developed herein for \( \text{SjAb} \) immunoassay have three main merits: (1) it only requires a relatively small total volume (i.e., 10 \( \mu \)l) of biomagnetic particle probes and samples or reagents and a relatively short assay time (i.e., 20 min) in a single analytical step; (2) the efficient mixing of a reactant suspension solution can be achieved with the use of an external low-rate mechanical shaking, leading to a “self-circulating flowing reactant system” that shows high antigen-antibody binding efficiency; and (3) it can be reused for over 300 measurements. Such a novel SSAP-based \( \text{SjAb} \) immunoassay is rapid, simple, specific, sensitive, and low-cost, and therefore holds great potential for clinical screening and diagnosis of schistosomiasis. Moreover, this lotus leaf-inspired self-cleaning SSAP can be extended as a new, universal, promising alternative platform to all other suspension assays such as AuNP-based colorimetric bioassays for more field-based bioapplications in resource-poor settings, including the diagnosis of infection diseases in developing countries, decentralized point-of-care testing, detection of biological warfare agents in the field, etc.

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Appendix A. Supplementary data

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