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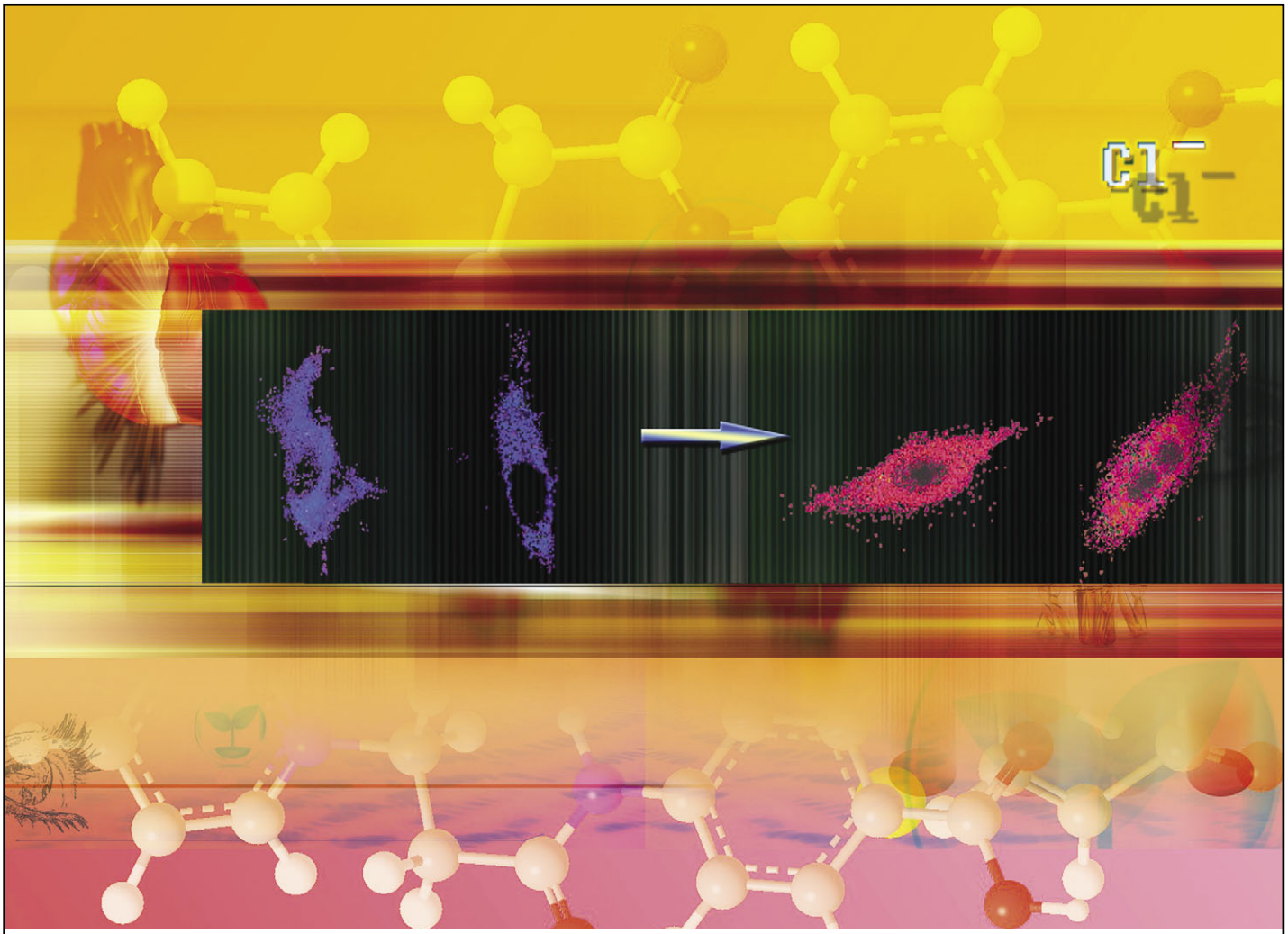

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Ratiometric fluorescence imaging for distinguishing chloride concentration between normal and ischemic ventricular myocytes

A new ratiometric fluorescent probe for Cl^- has been developed. Due to its excellent features, dynamic imaging of Cl^- has been achieved in ventricular myocytes during the myocardial ischemia course.

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COMMUNICATION

Ratiometric fluorescence imaging for distinguishing chloride concentration between normal and ischemic ventricular myocytes†

Ping Li, Ting Xie, Nannan Fan, Kexiang Li and Bo Tang*

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We devised a new ratiometric fluorescent probe for the detection of chloride ions. This synthesized probe was applied to the ventricular myocytes to successfully realize dynamic imaging of Cl^- concentration fluctuations during the myocardial ischemia course.

As an important anion in biosystems, chloride ions (Cl^-) are distributed widely in almost all kinds of cells. Cl^- ions are critically involved in many cellular functions,[1,2] such as the regulation of cell volume, cellular pH, intracellular traffic, immune response and apoptosis.[3,4] Anomalous fluctuations in the Cl^- level can result in some diseases,[5] such as cystic fibrosis,[6] myotonia[7] and arrhythmia.[8] Thus, the detection of Cl^- in biological systems has attracted extensive interest, and several approaches have been employed for monitoring Cl^- concentration.

It is notable that the Cl^- level in the heart has a close relationship with some heart diseases. Cl^- concentration in ventricular myocytes would increase dramatically under ischemic conditions,[9] which is clinically regarded as a feature of myocardial ischemia. Exploring a fast and convenient technique to monitor the fluctuations of Cl^- concentration in ventricular myocytes is of great value. Until recently, only the Cl^- double-barrel microelectrodes[9–11] were used in detecting the changes of Cl^- in myocardial ischemia. Presently, Cl^- sensing fluorescent probes reported are mostly designed based on a fluorescence-quenching mechanism,[4,12–17] which limits their applications to the biological system due to various interferences from an intricate biological environment. This issue could be easily conquered when the probe is designed as a ratiometric fluorescent probe.[18–22] Ratiometric fluorescence measurements not only can improve the sensitivity of the detection, but also can avoid interferences from background fluorescence. The reason is that the ratio of the fluorescent intensities at two wavelengths is independent of the probe concentration, the fluctuation of light-source intensity, and the sensitivity of instruments.[23] Therefore, this method is greatly useful for cellular imaging studies.[24]

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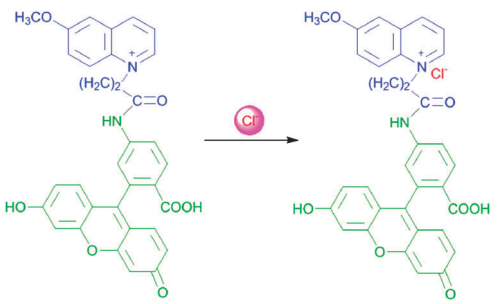

Scheme 1 The reaction of MQAF with Cl^-.

Herein, we developed a ratiometric fluorescent probe for sensing Cl^-. The probe, called MQAF (Scheme 1), is composed of two fluorophores, 5-amino-fluorescein (AF) and 6-methoxy-quinoline (MQMBP). AF serves as the Cl^- insensitive fluorophore. Meanwhile, MQMBP is chosen both as the other fluorophore and the Cl^- sensing group,[25] since its fluorescence intensity decreases linearly with the increased Cl^- concentration. We believe that the ratios of the dual emission wavelengths vary linearly with the increase of Cl^- concentrations.

The fluorescence responses of synthesized MQAF were studied by increasing Cl^- concentration gradually. When the probe was excited using the wavelength of 318 nm (exciting MQMBP moiety), the fluorescence intensity at 436 nm drops gradually with increased Cl^- concentration (Fig. 1a). Meanwhile, the fluorescence intensity of the AF moiety as the Cl^--insensitive group around 519 nm keeps nearly unchanged when the probe was excited at 494 nm (Fig. 1b). As expected, an excellent linearity between the fluorescence ratios (F_{519nm}/F_{436nm}) and Cl^- concentrations in the range of 0.5–100 mM was achieved (Fig. 1c), matching well with the Cl^- level in ventricular myocytes.[26] The regression equation is $F_{519nm}/F_{436nm} = 0.2523 + 0.01474[Cl^-]$ ($\times 10^{-3}$ M) with a linear coefficient of 0.9964, and the limit of detection was calculated to be 0.48 mM. These results indicate that our probe has the potential to detect Cl^- qualitatively and quantitatively.

When the concentration of the probe was kept at 8.0 μM and the concentrations of Cl^- were varied from 0 to 100 mM gradually, there is a linear relationship between F_0/F (fluorescence intensity at 436 nm with excitation at 318 nm) and concentrations of Cl^-, obeying the Stern–Volmer equation.[27] The linear approximation gives $F_0/F = 1.18 + 0.0598[Cl^-]$ with a linear coefficient of 0.9931 (Fig. 1d) and the slope shows that the Stern–Volmer

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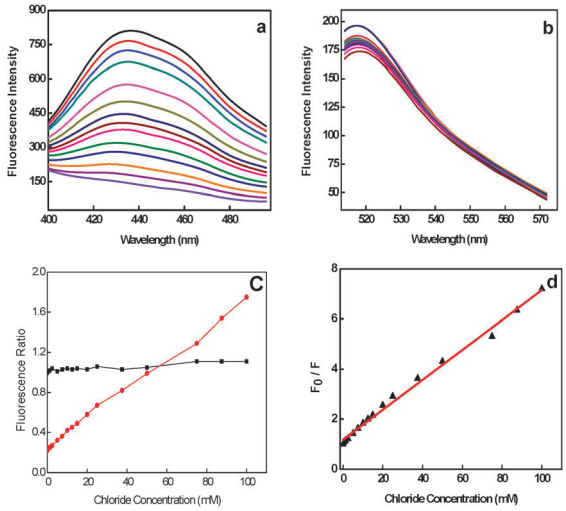

Fig. 1 Fluorescence responses of MQAF (8.0 μM) toward different concentrations of Cl^- in 20 mM HEPES buffer, pH 7.4. (a) Emission spectra of a MQMBP moiety of the MQAF with excitation at 318 nm, a linear change of fluorescence intensity *vs.* the Cl^- concentration is shown (final Cl^- concentration from top to bottom: 0.50, 1.25, 2.50, 5.00, 7.50, 10.0, 15.0, 20.0, 25.0, 37.5, 50.0, 75.0, 87.5, 100 mM). (b) Emission spectra of an AF moiety of the MQAF with excitation at 494 nm. (c) Plots of the fluorescence ratios F_{519nm}/F_{436nm} (red solid circles) *versus* Cl^- concentration yield a straight line relationship, and the ratios of F_{519nm}/F_{0519nm} (black solid squares) plotted in a similar way before and after Cl^- addition show a slope of zero, indicating no change in intensity over the tested range. F_{0519} and F_{519} denote the fluorescence intensities in the absence and in the presence of Cl^- at 519 nm, respectively. (d) The Stern–Volmer plot of the probe *versus* the Cl^- concentration. F_0/F are fluorescence ratios at 436 nm of the probe excited at 318 nm, F_0 and F denote the fluorescence intensities in the absence and in the presence of quencher Cl^-, respectively.

constant K_{SV} is 59.8 M^{-1}, and the dissociation constant was calculated to be 16.7 mM.

The interferences of the bio-relevant substance on monitoring Cl^- were also examined, and the experimental results are shown in Fig. 2. Although some anions, such as Br^- and I^-,

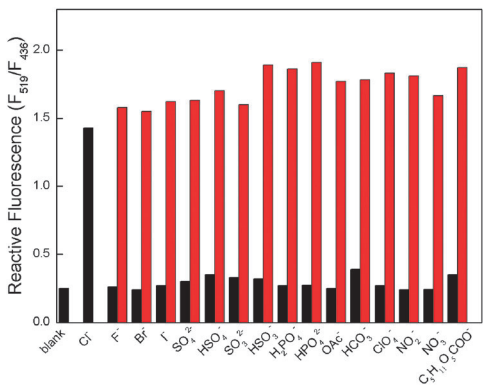

Fig. 2 Comparison of the fluorescent responses of the probe with various anions. The fluorescence intensities of the system were recorded when various anions (F^-, Cl^-, HCO^{3-}, SO_4^{2-}, $H_2PO_4^-$: 75 mM; NO^{3-}, HPO_4^{2-}, HSO_4^-, OAc^-, ClO_4^-, $C_5H_{11}O_5COO^-$: 25 mM; other anions: 1 mM for each) were introduced into 8.0 μM probe buffer solutions (20 mM, pH 7.4). Black bars only are various anions, and red bars represent the fluorescent response after addition of Cl^- to the mixture of other anions. All data were obtained at room temperature.

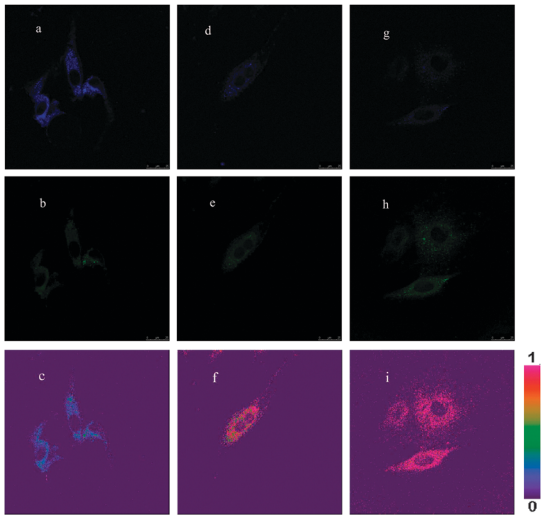

Fig. 3 Confocal fluorescence ratiometric imaging of ventricular myocytes in normal and ischemic ventricular myocytes. Cells incubated with 20 μM probe for 10 min at 37 °C were washed with HEPES buffer (20 mM, pH 7.4) three times before experiments. (a–c) blank group, (d–f) control group, (g–i) simulated ischemia group. Upper row presents fluorescence images with emission collected at 420–450 nm by the blue channel; middle row presents fluorescence images with emission collected at 510–540 nm by the green channel; lower row presents ratiometric images generated directly from the green channel and blue channel.

could also quench the fluorescence intensity of the MQMBP moiety to a certain extent, their concentrations are under a millimolar level in physiological conditions,[28–31] which are much lower than $[Cl^-]$.[32]

Concentrations of other anions used in the chemical selectivity experiment were all higher than their respective physiological concentrations. So they would not disturb the Cl^- detection in ventricular myocytes.

We applied MQAF to imaging of the ventricular myocytes to observe $[Cl^-]$ fluctuations, owing to the unique performance offered by MQAF. Ventricular myocytes (H9c2 (2-1)) were divided into three groups: blank group, control group and simulated ischemia group. The green fluorescence of the AF moiety is almost unchangeable when the probe was excited by 488 nm (Fig. 3b, e and h). At the same time, all the three groups give off a blue fluorescence of the MQMBP moiety under the excitation of 405 nm. But in comparison with the blank group, the blue fluorescence decreases dramatically in the control group (Fig. 3a and d). On the other hand, in the simulated ischemia group, the blue fluorescence intensity is decreased more than that in the control group (Fig. 3d and g), which is consistent with the ischemia-induced increase of the Cl^- concentration.[9] The fluorescent ratio of the blank group is the lowest (Fig. 3c), that of the control group increases (Fig. 3f) and that of the simulated ischemia group is the highest (Fig. 3i). Therefore, the ratio enhancement can reflect Cl^- concentration increase. These results demonstrated that fluorescence ratiometric imaging was successfully introduced to investigate the level of Cl^- in ventricular myocytes under normal or simulated ischemia conditions. It is also demonstrated that the concentration of $C1^-$ in ventricular muscle is

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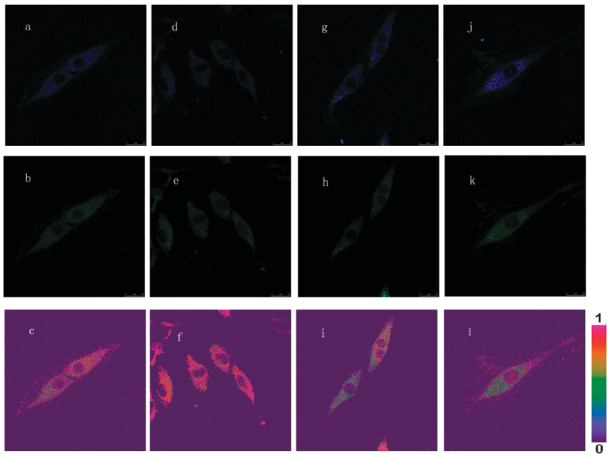

Fig. 4 Confocal fluorescence ratiometric imaging of living ventricular myocytes in different levels of myocardial ischemia. Incubation conditions were as in Fig. 3. (a–c) SITS group, (d–f) 50% Cl^- group, (g–i) 25% Cl^- group, (j–l) Cl^--free group. Upper row presents fluorescence images with emission collected at 420–450 nm by the blue channel; middle row presents fluorescence images with emission collected at 510–540 nm by the green channel; lower row presents ratiometric images generated directly from the green channel and blue channel.

increased under ischemic conditions, which might be attributed to the activation of the Cl^-–HCO_3^- exchanger.[26]

It is established that applications of the Cl^- channel blocker such as 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonicacid (SITS, a stilbene derivative)[33,34] or reducing the extracellular Cl^- concentration would delay the onset of ischemia induced elevation in Cl^- concentration,[5] and thus could alleviate the symptom of myocardial ischemia reperfusion injury.[35] We then examined the Cl^- concentration alteration in different levels of myocardial ischemia treated by SITS or reduction of extracellular Cl^- and the images are displayed in Fig. 4. Compared with the simulated ischemia group shown in Fig. 3, the blue fluorescence intensity recovers to some extent (Fig. 4a) after application of SITS, which could reduce the up-taken amount of Cl^-. Fig. 4c represents the ratiometric image of the SITS group which indicated the reduction. When concentrations of the extracellular Cl^- were decreased to 50%, 25% and 0, respectively, corresponding changes occurred in the ratiometric images (Fig. 4f, i and l), which indicated that MQAF could respond to the concentration fluctuation of Cl^-.

In conclusion, a new ratio fluorescent probe designed could serve as a new potent tool for detecting Cl^- at the cellular level. The probe is stable and can respond instantaneously to the fluctuation of Cl^- concentration. Importantly, it realizes the dynamic, visualizable analysis of Cl^- level difference between normal and ischemic ventricular myocytes, for the first time. Hence, the ratiometric fluorescence method reported herein contributes to shed new light on Cl^- detection *in vivo*. Moreover, it would hold considerable promise in the investigation of other biological anion species-mediated cellular behaviors.

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