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Immunosensor for Rapid and Sensitive Detection of Digoxin

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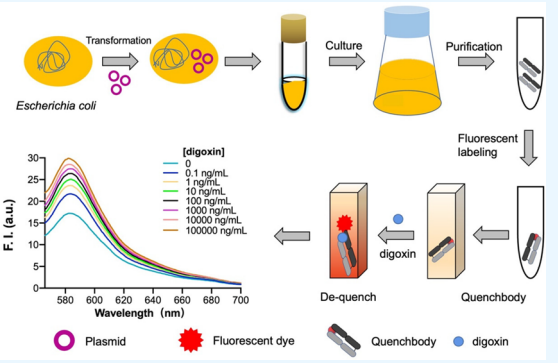
ABSTRACT: Digoxin is a cardiac glycosylated steroid-like drug with a positive inotropic effect and has been widely used in treating congestive heart failure, atrial fibrillation, atrial flutter, and other heart diseases. Digoxin is also a dangerous drug, which can cause drug poisoning at a low blood drug concentration (2.73−3.9 nmol/L, i.e., 2.14−3.05 ng/mL). Therefore, the timely detection of a patient's blood drug concentration plays a significant role in controlling blood drug concentration, reducing the occurrence of drug poisoning events, and maximizing the role of drug therapy. In this study, a DNA vector for the expression of the antidigoxin antibody Fab fragment was constructed. With the vector, Fab was expressed in *E. coli* and purified, and 1.2 mg of antibodies was obtained from 100 mL of culture. An immunofluorescent sensor based on the mechanism of photoinduced electron transfer was constructed by labeling additional cysteines in the heavy chain variable region and light chain variable region of the antibody Fab fragment with fluorescent dyes. The assay for digoxin with the immunosensor could be finished within 5 min with a limit of detection of 0.023 ng/mL, a detectable range of 0.023 ng/mL to 100 μg/mL, and an EC_{50} of 0.256 ng/mL. A new approach for the rapid detection of digoxin was developed and will contribulte to therapeutic drug monitoring.



1. INTRODUCTION

Since the 1990s, the overall incidence of heart disease has been increasing, seriously threatening the life and health of patients.[1] Digoxin is a cardiac glycosylated steroid-like drug with a positive inotropic effect (Figure 1a) that is widely used in treating congestive heart failure, atrial fibrillation, atrial flutter, and other heart diseases.[2] The large individual differences and narrow safety range of digoxin are its most prominent pharmacokinetic characteristics; the narrow safety range increases the difficulty of controlling the therapeutic dose to prevent poisoning.[3] Digoxin is also a dangerous drug, which can cause drug poisoning at a low blood drug concentration (2.73−3.9 nmol/L, i.e., 2.14−3.05 ng/mL), and the dose causing adult mortality is as low as about 10 mg. Therefore, the timely detection of a patient's blood drug concentration plays a significant role in controlling blood drug concentration, reducing the occurrence of drug poisoning events, and maximizing the role of drug therapy. Detecting the serum drug concentrations is of great significance for maintaining the effective therapeutic dose of drugs and minimizing the possibility of drug poisoning. The existing monitoring methods mainly include high-performance liquid chromatography (HPLC),[4] fluorescence polarization immunoassay,[5] liquid chromatography−mass spectrometry (LC-MS),[6] chemiluminescence immunoassay, microparticle enzyme-linked immunoassay,[7] and radioimmunoassay.[8] However, these methods have the disadvantages of complex operation, a long time for completion, and a high detection cost. Quenchbody (Q-body) is an antibody or an antibody fragment labeled with fluorescent dye(s). The fluorescent dyes labeled on the antibody enter the inside of the antibody, and their fluorescence is quenched by tryptophans in the antibody via photoinduced electron transfer. However, when Q-body binds to its antigen, the electron transfer is relieved and the fluorescence is restored. By measuring the change of fluorescence intensity, the antigen can be determined easily and quickly.[9] Q-bodies based on fluorescence quenching have the advantages of simple operation and rapid detection. So far, the Q-body assay has been used for detecting bone gla protein,[10] aldosterone,[11] estradiol,[12] and the S protein of SARS-CoV-2.[13] In this study, a Q-body was developed for the rapid detection of digoxin for the first time, and it may have great significance in clinical applications.

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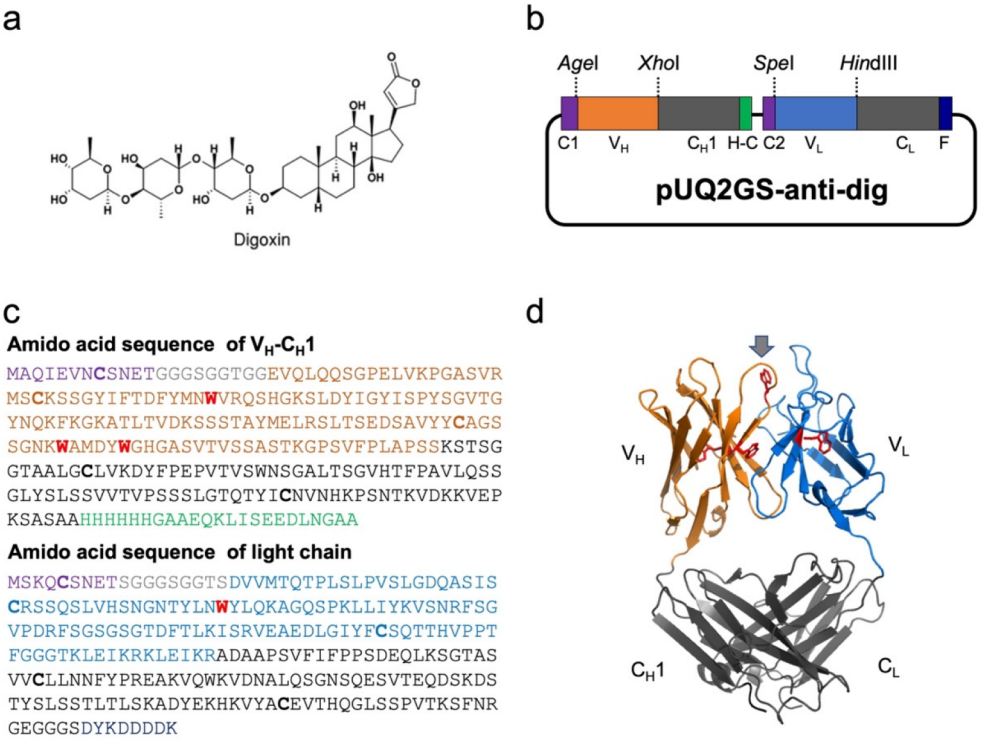

Figure 1. Preparation of antidigoxin Fab antibody. (a) Molecular structure of digoxin. (b) Construction of protein-expressing vector, pUQ2GS-anti-dig. (c) Amino acid sequence of designed V_H−C_H1 and light chain. Cysteines and tryptophan residues in V_H−C_H1 and light chain are shown in bold. (d) Structure of 26−10 Fab. C1 (purple), MAQIEVNCSNET; V_H (orange), variable region of antibody heavy chain; C_H1 (gray), constant region 1 of human IgG; H−C (green), his and c-myc tag, HHHHHHGAAEQKLISEEDLNGAA; C2 (purple), MSKQCSNETS; V_L (cyan), variable region of antibody light chain; C_L (gray), kappa region of human IgG; F (blue), Flag-tag, DYKDDDDK. Arrow indicates the antigen-binding activity of Fab. Tyrptophans are highlighted in red and shown in sticks.

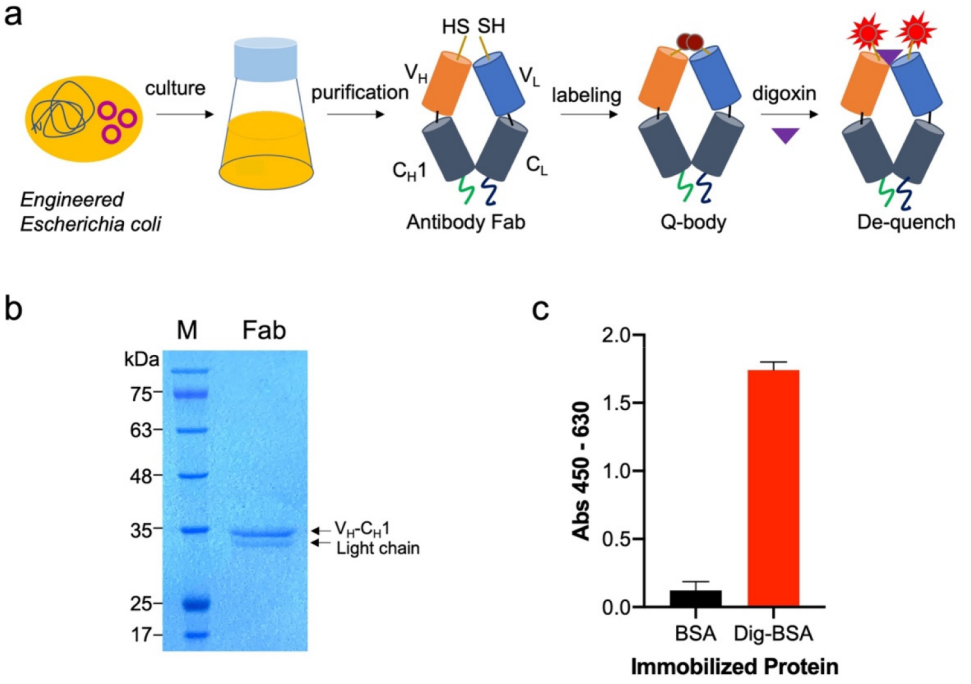

Figure 2. Preparation of antidigoxin Fab antibody. (a) Scheme for preparation of Q-body for digoxin detection with Fab. (b) SDS-PAGE analysis of expressed antidigoxin Fab. M: Biostep Prestained Protein Marker (Tanon, Shanghai, China). (c) ELISA result of the antigen-binding activity of purified Fab. BSA, bovine serum albumin; Dig-BSA, conjugate of BSA and digoxin.

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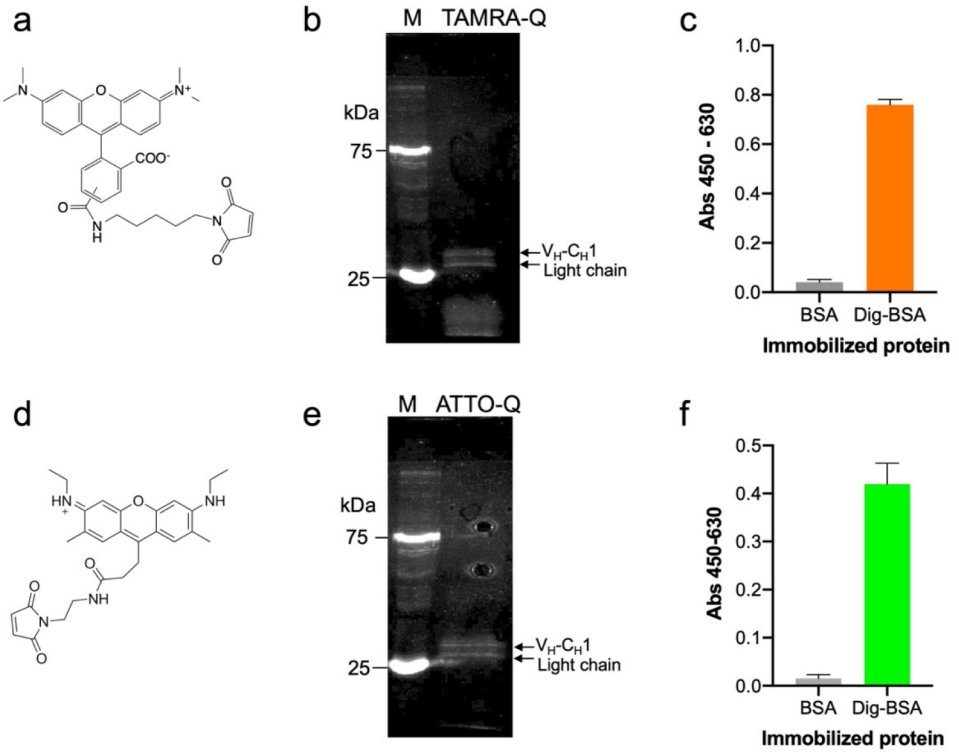

Figure 3. Preparation of Q-bodies. (a) Structure of TAMRA-C5-maleimide. (b) SDS-PAGE analysis of TAMRA-labeled Fab (TAMRA Q-body). (c) ELISA analysis of the antigen-binding activity of TAMRA Q-body. (d) Structure of ATTO520-C2-maleimide. (e) SDS-PAGE analysis of ATTO520-labeled Fab (ATTO520 Q-body). (f) ELISA analysis of the antigen-binding activity of the ATTO520 Q-body. M: Precision Plus Protein Dual Color Standards (Bio-Rad).

2. RESULTS AND DISCUSSION

2.1. Design and Construction of Digoxin Q-Bodies. The genes of the heavy chain variable region (V_H) and light chain variable region (V_L) of the antidigoxin antibody 26−10[14] were amplified with the polymerase chain reaction (PCR), and agarose gel electrophoresis was performed to confirm the PCR product. Single and obvious nucleic acid bands were observed at 300−400 bp and were identified as V_H and V_L genes. After recovery and purification, the DNA fragments was treated with the restriction enzymes *Age*I and *Xho*I. The vector pUQ2GS[10] for expression of the antigen-binding fragment (Fab) of the antibody was also treated with *Age*I and *Xho*I, and a linearized vector with sticky ends was separated by agarose gel electrophoresis and purified. The treated plasmid and V_H were enzymatically linked and transformed into *Escherichia coli* DH5α competent cells, and single colonies grown on solid culture medium were identified. pUQ2GS with the antidigoxin antibody V_H was screened and treated with restriction endonucleases *Hin*dIII and *Spe*I to ligate with V_L that was treated with the same enzymes. After colony PCR and sequence analysis, the expression vector pUQ2GS-anti-dig for expressing Fab of the antidigoxin antibody was obtained (Figure 1b). The designed Fab amino acid sequence is shown in Figure 1c. pUQ2GS-anti-dig expresses Fab with a peptide C1 containing a cysteine (MAQIEVNCSNET) at the N terminus of V_H, a peptide C2 (MSKQCSNET) at the N terminus of V_L for fluorescent dye labeling, a His-tag at the C-terminus of Fd (V_H−C_H1), and a Flag-tag at the C-terminus of the light chain (V_L−C_L) for purification. The structure of 26−10 Fab modified from PDB 1IGI is shown in Figure 1d. The two cysteines attached to the N termini of the V_H and V_L of the Fab are on the outside of the Fab stereostructure, while each pair of cysteines in the V_H, C_H1, V_L, and C_L are inside of Fab and form disulfide bonds to make Fab stable. The two additional cysteines may form disulfide bonds with each other. TCEP at a relatively low concentration was used to open them and make them react with maleimide fused to fluorescent dyes, while they do not affect the internal disulfide bonds.

2.2. Expression and Purification of Fab of Antidigoxin Antibody in *E. coli*. The scheme for preparation of Q-body and detection of digoxin was shown in Figure 2a. *E. coli* SHuffle T7 Express lysY strain was transformed with pUQ2GS-anti-dig and cultivated followed by additaiont of Isopropylthio-β-galactopyranoside (IPTG) for induction of Fab expression. The expressed Fab was purified using Ni-NTA Sefinose Resin and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After coomassie brilliant blue (CBB) staining, two protein bands were observed. The molecular weight of the upper band was approximately 30 kDa and that of the lower band was approximately 28 kDa (Figure 2b), which were slightly different from the theoretical molecular weights of 28 kDa and 26 kDa, respectively. However, because of the high purity of the protein solution, the two bands were considered to be the V_H−C_H1 and the light chain (V_L−C_L), respectively. The difference in the molecular weight of the protein bands may be caused by differences in amino acid composition and electrophoresis conditions. Quantitative analysis showed that 1.2 mg of purified Fab could be obtained per 100 mL of *E. coli* culture.

2.3. Detection of Digoxin-Binding Activity of Purified Fab. An enzyme-linked immunosorbent assay (ELISA) was performed to confirm the antigen-binding activity of Fab with bovine serum albumin (BSA) and conjugate of BSA and digoxin (Dig-BSA) coated in 96-well plates. The assay was performed by

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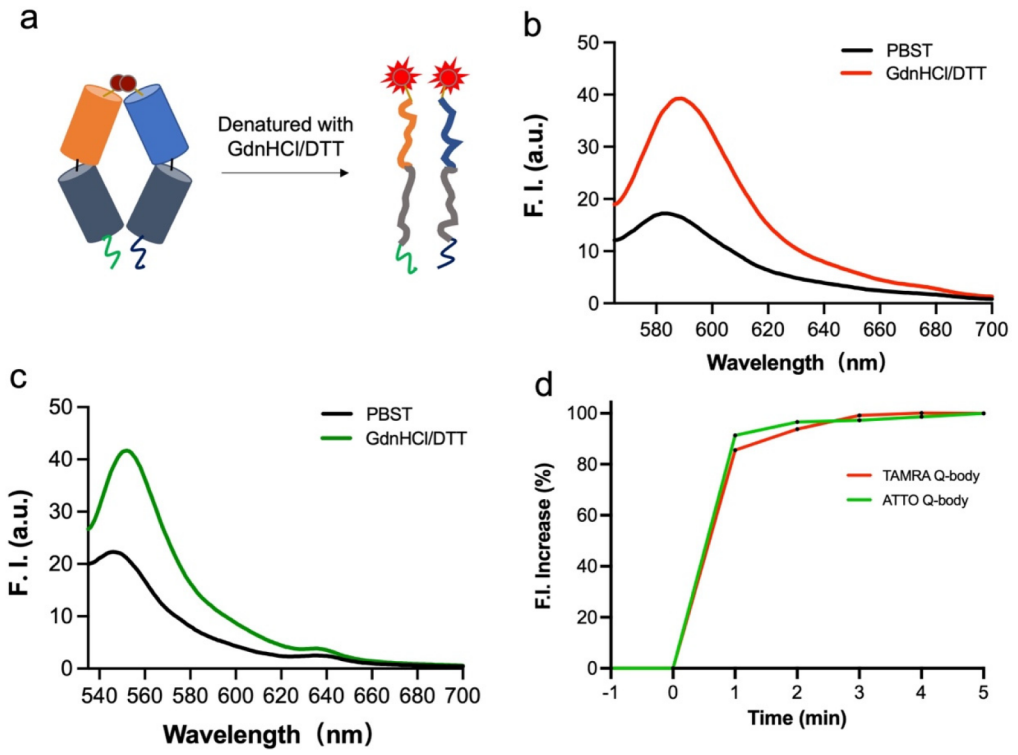

Figure 4. Characterization of Q-bodies. (a) Scheme of denaturation of TAMRA Q-body with GdnHCl/DTT. (b) Fluorescent spectrum of TAMRA Q-body while denatured. (c) Fluorescent spectrum of ATTO520 Q-body while denatured. (d) Time course for determination of digoxin with Q-bodies. GdnHCl/DTT: 7 M guanidine hydrochloride solution containing 100 mM dithiothreitol.

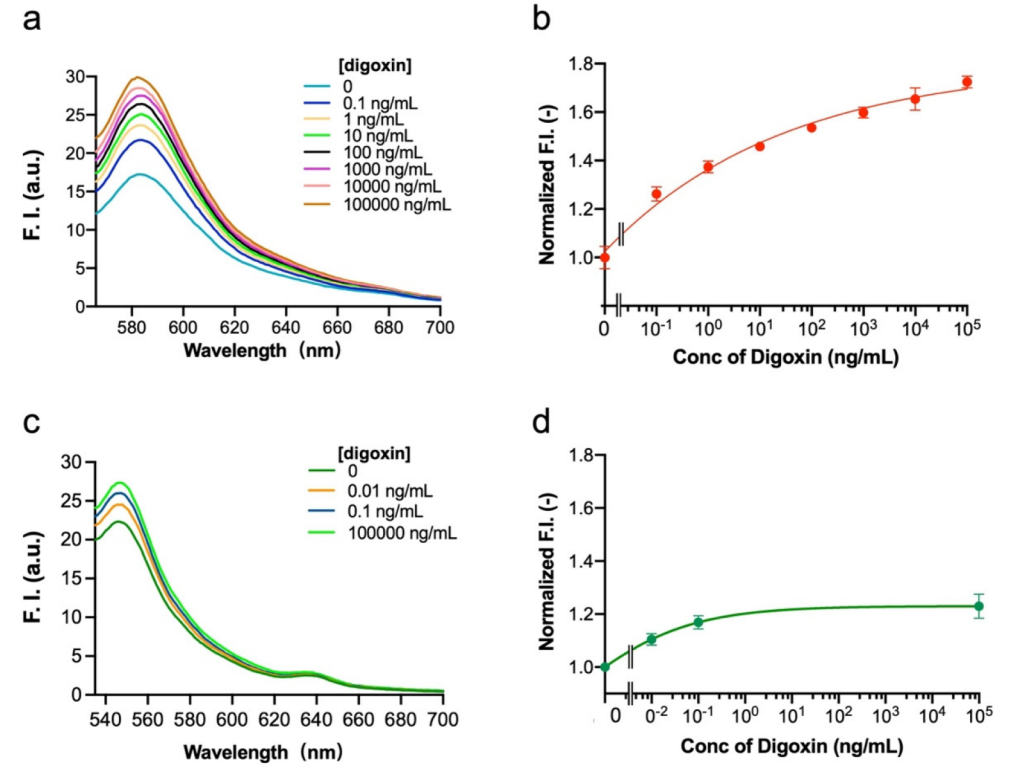

Figure 5. Detection of digoxin with Q-bodies. (a) Fluorescence spetra of TAMRA Q-body in the presence of digoxin. (b) Dose−response curve for detection of digoxin with TAMRA Q-body. (c) Fluorescence spetra of ATTO Q-body in the presence of digoxin. (d) Dose−response curve for detection of digoxin with ATTO520 Q-body. F.I.: Fluorescence intensity. Error bars represent standard errors of triplicate samples.

the addition of Fab, a secondary antibody, and horseradish peroxidase (HRP)-labeled antibody; incubation; and color development. As shown in Figure 2c, the average absorbance of the sample wells coated with Dig-BSA was 1.74, whereas that of

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the BSA-coated wells was 0.12, and the signal-to-noise ratio was calculated to be 14.5. These results indicated that the antidigoxin Fab antibody produced by prokaryotic expression has specificity and binding activity to digoxin.

2.4. Preparation of Q-Bodies for Digoxin Detection. The Fab purified with Ni-NTA beads was labeled with the fluorescent dye TAMRA-C5-maleimide (Figure 3a) by a thiol-maleimide reaction. After labeling, another purification step with anti-DYKDDDDK beads was conducted, and the eluate was analyzed by SDS-PAGE. As shown in Figure 3b, a fluorescent band was observed at approximately 28 kDa, which was consistent with the molecular weight of V_H-C_H1 of Fab. Another band observed at 26 kDa was considered to be the light chain. The results suggested that the labeling was successful, and a TAMRA-C5 labeled Q-body (TAMRA Q-body) was obtained. ATTO520-C2-maleimide (Figure 3d) was also used for labeling Fab to prepare the ATTO520 Q-body (ATTO Q-body), and two fluorescent bands, Fd and the light chain of ATTO Q-body, were observed after SDS-PAGE, as shown in Figure 3e.

2.5. Determination of Antigen-Binding Activity of Q-Bodies. The antigen-binding activities of the TAMRA and ATTO520 Q-bodies were investigated using ELISA. The average absorbance of the TAMRA Q-body bound to Dig-BSA after fluorescence modification and flag purification was 0.76, while the absorbance of TAMRA Q-body-bound BSA was 0.041 and the signal-to-noise ratio was 18.5, which indicated that the TAMRA Q-body retained its antigen binding activity (Figure 3c). The absorbance values of the ATTO520 Q-body bound to Dig-BSA and BSA were 0.419 and 0.015, respectively, and the signal-to-noise ratio was 27.9 (Figure 3f). These results suggested that both Q-bodies have antigen-binding activity of the original antidigoxin antibody.

2.6. Detection of Digoxin with Q-Bodies. To identify that the fluorescence of dyes labeled to the Fab were quenched intramolecularly and that prepared Q-bodies have the capacity for antigen detection, we used 7 M guanidine hydrochloride solution containing 100 mM dithiothreitol (GdnHCl/DTT) as a denaturant solution to destroy the Fab antibody structure and its disulfide bond, so that the fluorescence of the Q-body fluorescent dye can be maximally released (Figure 4a). The experimental results for the TAMRA Q-body are shown in Figure 4b. The fluorescence value obtained with the denaturant was 2.2-fold higher than that of phosphate buffer saline (PBS) containing 0.5% Tween 20 (PBST), indicating that the fluorescence of TAMRA was quenched in the antibody Fab fragment. Similar to the TAMRA Q-body, the fluorescence value of the ATTO520 Q-body obtained with the denaturant was 1.96-fold higher than that obtained with PBST (Figure 4c), indicating that the ATTO520 Q-body also exhibited fluorescence quenching. A time course of Q-body for the detection of digoxin was tested, and as shown in Figure 4d, both Q-bodies reached the maximum fluorescence intensity within 5 min, which allows the detection of digoxin be carried out in 5 min during the subsequent experiments.

After confirming the fluorescence quenching of the Q-body, digoxin with different concentration gradients was added to the PBST solution containing the TAMRA Q-body, and the fluorescence spectra are shown in Figure 5a. With the increase in antigen concentration, the fluorescence intensity of the sample gradually increased, and the fluorescence value at 585 nm increased 1.72-fold at the highest antigen concentration compared to that without antigen, while the change in the fluorescence value of the ATTO520 Q-body was only 1.22-fold (Figure 5c).

Figure 5b shows the dose−response curve drawn based on the fluorescence intensity at 585 nm for digoxin detection using the TAMRA Q-body. The TAMRA Q-body assay had a limit of detection (LOD) of 0.023 ng/mL, a half-maximum effective concentration (EC_{50}) of 0.256 ng/mL, and a detection range of 0.023 ng/mL to 100 μg/mL. However, the LOD and EC_{50} of the assay with the ATTO520 Q-body were not calculated because the fluorescence increase was limited, as shown in Figure 5d.

2.7. Detection of Digoxin in Spiked Human Serum. To verify that the prepared Q-body can be used to detect digoxin in serum, human serum samples spiked with 1.5, 5.0, and 150.0 ng/mL digoxin were used for recovery experiments. As shown in Table 1, the detected concentrations were 1.3, 4.2, and 131.8 ng/mL with recovery rates of 86.7%, 84%, and 87.9%, respectively. The detected concentrations were slightly lower than the actual concentration. However, it can be improved by further optimizing the experimental conditions when developing kits for clinical use. The above results suggested its potential in clinical application.

Table 1. Recovery of Spiked Digoxin in Human Serum Sample

spiked conc. (ng/mL)	measured conc. (ng/mL)	recovery rate (%)
1.5	1.3	86.7
5.0	4.2	84.0
150	131.8	87.9

Digoxin is a digitalis-derived cardioside that is often used to treat heart failure, arrhythmias, and other cardiovascular-related diseases. Its role in the treatment of cardiovascular-related diseases is to increase myocardium contractility to increase blood pressure and reduce the heart rate of patients. Recent studies also showed that digoxin could be used for geroprotection in patients with frailty and multimorbidity.[15] Although it is a therapeutic drug, the narrow therapeutic window makes it a less safe drug. The individual difference in drug use is large, and it can pass through the placenta and the blood−brain barrier. The therapeutic dose is approximately 3/5th of the toxic dose. In patients with hypokalemia and hypomagnesemia, the toxic dose is closer to the therapeutic dose.[16,17] Common manifestations of toxic reactions are nausea, vomiting, hypotension, and some nonspecific clinical manifestations that are difficult to find. In severe cases, shock and cardiac arrest may occur, threatening the life and health of patients.[18] Relevant investigation results show that when the blood drug concentration of patients exceeds 2.0 ng/mL, monitoring should be strengthened and reasonable measures should be taken in time to prevent or ameliorate drug poisoning reactions.[19] Therefore, timely, effective, and accurate blood drug concentration detection methods play an important role in improving drug safety and optimizing patient prognosis.

Methods for detecting digoxin concentration in blood mainly include HPLC,[20] capillary electrophoresis, LC-MS, and immunoassays including radioimmunoassay, enzyme immunoassay, fluorescence immunoassay, and chemiluminescence immunoassay.[21] Some of them are summarized as Table 2. HPLC can effectively exclude the influence of digoxin metabolites in patients on the detection results and has high specificity for digoxin.[22−24] The results obtained using this method were accurate and highly sensitive. However, it has a

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Table 2. Comparison of Detection Approaches for Digoxin

detection method	principle or steps	limit of detection	advantages and disadvantages
HPLC[4]	high-pressure infusion system; the mobile phase is pumped into the stationary phase; intracolumn separation; and postseparation detection	0.72 ng/mL	highly sensitive but expensive equipment and maintenance are needed
fluorescence polarization immunoassay[5]	fluorescence polarization technology and competitive binding of antigen/antibody	2 ng/mL	convenient, fast but limited to the small molecules, low sensitivity, and specific antibodies required
DNA-based digoxin detection[27]	changes in the equilibrium of the DNA strand displacement competition response	7.8 μg/mL	convenient but low concentrations of digoxin need to be detected over longer time
lateral flow immunoassay with multicolor gold nanorod etching[28]	the sample acts through a capillary tube and is visually detected with a colorimetric index bound to the antibody	not reported	convenient, fast but samples require additional processing steps and can only be semiquantitative
HPLC-tandem mass spectrometry[20]	ionizing components of the substance, the mass-to-charge ratio of the ions generated by different components, and qualitatively analyzed	0.5 ng/mL	highly sensitive but the equipment and reagent are expensive; highly skilled operator is needed
Q-body technology (this study)	detection based on the photoinduced electron transfer between fluorescent dye and tryptophan of antibody	0.023 ng/mL	convenient, fast and highly sensitive; preparation of recombinant antibody and labeling are needed

Table 3. Primers Used in This Study

primer name	sequence (5′−3′)	length (bp)
AgeIDigxinVHback	GGTGGAACCGGTGGTGAGGTACAGTTGCA	29
XhoIDigoxinVHfor	GCGCTCGAGACAGTAACGGACGCTCCGT	28
SpeIDigoxinVLback	GGTACTAGTGATGTGGTGATGACACAGACT	30
HinIIIDigoxinVLfor	GATTTCAAGCTTGCGCTTAATCTCCAGTTTG	31
T7 promoter	TAATACGACTCACTATAGGG	20
T7 terminator	GCTAGTTATTGCTCAGCGG	19

certain requirement for operational technology,[25] and thus this method is preferred when the sample size is small. Radioimmunoassay (RIA) combines the high sensitivity of radionuclide tracing technology and the high specificity of immunochemistry. Although its economic cost is relatively low, there are problems such as radioactive pollution, short half-life of markers, and long detection time. The enzyme immunoassay based on the improvement of RIA uses enzymes for antigen labeling, causing no radioactive pollution and extending the marker's half-life. However, enzymes are vulnerable to pH and temperature changes, and human factors have a significant impact on the operation process. Li et al. developed a method for the quantitative detection of digoxin in plasma based on the principle of competitive immunosorbent assay.[26] However, it requires high-quality antibodies and is prone to generating nonspecific background signals. Besides the above-mentioned conventional detection methods, researchers have developed a homogeneous binding analysis method to avoid a series of cumbersome steps such as fixation, separation, and washing.[27] Owing to the heterogeneity of conventional methods, the methods often take time to determine blood drug concentration and require fixation of conjugates on the surface and complicated operating procedures.

In this study, a novel method for detecting digoxin content based on a photoinduced electron transfer mechanism with the aid of an immune reaction was developed. Its advantages are that it does not require cumbersome instruments; avoids the impact of fixation, washing, and other operations on the experimental results; and is rapid and highly sensitive. We created a prokaryotic expression vector that stably expressed the soluble Fab fragment of the antidigoxin antibody in *E. coli*. By lowering the cultivation temperature to avoid the Fab inclusion body production, a relatively pure and real amount of protein was obtained through prokaryotic expression purification. We fluorescently labeled and purified the antibody solution and constructed an immunofluorescence sensor for digoxin detection. The assay with Q-body prepared with TAMRA-C5-maleimide exhibited a sensitivity of 0.023 ng/mL and a detection range of 0.023 ng/mL to 100 μg/mL, with the assay being sufficiently sensitive to measure the poisonous serum digoxin concentration of patients taking drugs. However, the Q-body prepared by ATTO520-C2-maleimide was not ideal for detection, which may be due to the insufficient length of the linker and the failure to send the dye to the optimal position. The lateral-flow immunoassay is also a rapid detection technology. Recently, great progress has been made in improving the detection sensitivity of COVID-19.[29,30] A combination of Q-body with the above approaches may create more excellent detection technologies in the future.

3. CONCLUSIONS

A new approach for rapid detection of digoxin with high sensitivity was developed. The operation of the established method is simple and rapid. It can provide data in time as a basic tool for monitoring the digoxin content in patients' blood and help doctors decide further treatment plans.

4. MATERIALS AND METHODS

4.1. Materials. *E. coli* competent DH5α cells for gene cloning and plasmid amplification were purchased from Shenzhen Kangti Life Science (Shenzhen, China), and *E. coli* SHuffle T7 express lysY from NEB[31,32] was used for Fab expression of antidigoxin antibody. Plasmid extraction and gel recovery kits were purchased from Vazyme Biotechnology Co., Ltd. (Nanjing, China). The restriction enzyme used for constructing the recombinant plasmid was purchased from New England Biolabs (NEB; Ipswich, MA, USA). The Ni-NTA Sefinose Resin for protein purification was purchased from Changzhou Smart-Lifesciences Biotechnology Co., Ltd. (Changzhou, China), and Dig-BSA was purchased from Wuhan Yunclon Technology Co., Ltd. (Wuhan, China). ATTO520-C2-maleimide was obtained from ATTO-TEC (Siegen, Germany) and TAMRA-C5-maleimide was obtained from Biotium (Hayward, CA, USA). Other reagents, if not specified, were purchased from Sigma-Aldrich, Shanghai Sango Biotechnology, and Beijing Solarbio Biotechnology Co. Ltd.

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(Beijing, China). Primers used in this study were synthesized by Shanghai Sangong Biotechnology Co., Ltd. (Shanghai China).

4.2. Amplification of Digoxin Antibody Gene. The gene of the antidigoxin antibody based on 26−10 was synthesized by Shanghai Sango Biotechnology Co., Ltd. after optimizing the design based on reference data,[33] and the gene was inserted into the pUC57 vector. The V_H gene of the digoxin antibody was amplified by PCR with primers AgeIDigoxinVHback and XhoIDigoxinVHfor, while V_L was amplified with primers SpeIDigoxinVLback and HinIIIDigoxinVLfor using the pUC57-inserted antibody gene as a template. The primers used in this study are shown in Table 3. The DNA polymerase used for PCR was KOD-plus-Neo (Toyobo Co. Ltd., Osaka, Japan). The reaction conditions for the amplification of the V_H and V_L genes were as follows: Incubation at 98 °C for 2 min was carried out first to predenature the DNA, and then denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, and extension at 68 °C for 1 min was carried out for 30 cycles. The PCR products were recovered according to the manufacturer's instructions (Vazyme, Nanjing, China). The V_H gene and pUQ2GS were treated with the restriction enzymes *Age*I and *Xho*I at 37 °C for 5 h. After enzyme digestion, agarose gel electrophoresis was performed, and the separated V_H gene was recovered using a DNA recovery kit (Vazyme). Ligation high ver.2 (Toyobo) was used to ligate the treated V_H gene and the digested pUQ2GS vector at 16 °C for 2 h. The ligation mixture was then used to transform *E. coli* DH5α competent cells, and cells were plated on an LB plate containing 100 μg/mL of ampicillin (LBA) and cultured upside down in a 37 °C incubator for 18 h. Single colonies were picked up after the culture, and colony PCR with primers T7 promoter and XhoIDigoxinVH for was carried out to screen the colonies containing the antidig V_H plasmid. Selected positive colonies were cultivated in 4 mL of LB liquid medium for 12 h, followed by plasmid extraction and sequence analysis to obtain the pUQ2GS-VH recombinant plasmid containing V_H. pUQ2GS-VH and the PCR-amplified V_L gene fragment were digested with *Spe*I and *Hin*dIII and ligated using a protocol similar to that described above. Positive colonies were screened using the primers SpeIDigoxinVLback and T7 terminator, and the recombinant plasmid pUQ2GS-anti-dig, which can express the Fab fragment of antidigoxin Fab, was obtained.

4.3. Expression and Purification of Digoxin Antibody. *E. coli* SHuffle T7 Express lysY was transformed with pUQ2GS-anti-dig, spread on an LBA plate, and cultured upside down at 37 °C for 12 h. After the culture, single colonies were selected and cultured in 4 mL of liquid medium containing ampicillin overnight by shaking at 37 °C and 250 rpm for 12 h. Then, the obtained bacterial solution was inoculated into 300 mL of LBA liquid medium and cultured in a shaking incubator at 37 °C until the OD value at 600 nm reached 0.4. IPTG with a final concentration of 0.4 mM was added to induce protein expression at 16 °C with shaking at 150 rpm for 22 h. The culture solution was centrifuged at 8000*g* for 5 min at 4 °C, resuspended with 10 mL of TALON extraction buffer (8 mM Na_2HPO_4, 47.9 mM NaH_2PO_4, and 300 mM sodium chloride, pH 7.0), and then sonicated on ice for 15 min with 2 s working and 3 s interval cycles. After sonication, centrifugation was carried out at 10 000*g* for 10 min at 4 °C, and the supernatant was collected and purified with Ni-NTA Sefinose Resin to obtain the purified Fab fragment of the digoxin antibody. The purified Fab was analyzed using SDS-PAGE.

4.4. Identification of Antibody Activity. The antigen-binding activity of Fab was determined using an ELISA. One-hundred microliters of 2 μg/mL BSA and Dig-BSA was coated on a microplate and incubated at 4 °C for 12 h. The next day, the plate was washed thrice with PBST and then blocked with 2% fresh skimmed milk in PBS (MPBS) at 25 °C for 2 h. After blocking, the plate was washed with PBST three times, and 2 μg/mL expressed antidigoxin antibody Fab fragment was added and incubated at 25 °C for 1 h. The secondary antibody rabbit anti-Igκ chain was added and incubated for 1 h at 25 °C, followed by washing five times and the addition and incubation of HRP-conjugated goat antirabbit antibody diluted 1:4000 at 25 °C for 1 h. The plate was washed with PBST again, and 100 μL of TMBZ (3,3′,5,5′-tetramethylbenzidine at 0.2 mg/mL in 100 mM sodium acetate at pH 6.0 with 1:10000 diluted 30% H_2O_2) was added to the wells. The absorbance of the wells at 450 nm and 630 nm was measured on an iMark microplate reader (Bio-Rad, Heracules, CA, USA) after terminating the reaction with 50 μL of 10% H_2SO_4.

4.5. Preparation and Activity Detection of Digoxin Q-Body. Fifty micrograms of antidigoxin Fab antibody in a centrifuge tube was added to tris(2-carboxyethyl) phosphine (TCEP) at a final concentration of 0.5 mM and incubated at 4 °C in the dark for 20 min. Then, 4-azidobenzoic acid at a final concentration of 2 mM was added to the tube and incubated on ice for 10 min to stop the TCEP action. Subsequently, TAMRA-C5-maleimide or ATTO520-maleimide at 10-fold the amount (molar ratio) of Fab was added and incubated with rotation for 2 h in the dark. Then, 50 μL of anti-DYKDDDDK magnetic beads (GenScript, Nanjing, China) was added to the reaction solution and incubated for 2 h in the dark. The magnetic beads were washed five times with TBST (Tris-buffered saline with 0.1% Tween20) with 0.1% Birij to remove free fluorescent dye. Finally, elution buffer (25 μL of 3 × Flag peptide solution with 125 μL TBS solution) was added to the tube and incubated at 4 °C for 30 min, followed by centrifugation at 9000*g* for 1 min. The eluent's purity was confirmed by SDS-PAGE, and antigen-binding activity was confirmed by ELISA.

4.6. Detection of Digoxin with Q-Bodies. To test the detection time for digoxin, 10 ng of each purified Q-body was added to 1 mL of PBST in a 5 × 5 mm quartz cuvette. The fluorescence intensity was measured 1 min before digoxin was added at 100 μg/mL, and every other minute after the addition of digoxin until 5 min on a fluorescence spectrophotometer (Model F-4600, HITACHI, Tokyo, Japan). The excitation wavelength for TAMRA-labeled Q-bodies was 546 nm, and that for ATTO520-labeled Q-bodies was 520 nm, with slit widths of 5.0 nm. One microliter of PBST or GdnHCl/DTT was added to the two groups of cuvettes, respectively, in which 10 ng of digoxin Q-body was added. After incubation at 25 °C for 5 min, the fluorescence spectrum was detected. Furthermore, digoxin was added at final concentrations of 0 to 100 000 ng/mL for TAMRA Q-body and 0 to 100 000 ng/mL for ATTO520 Q-body, and the fluorescence spectrum at each antigen concentration was measured. Data were processed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). The fluorescence intensity at 585 nm for each antigen concentration was selected to obtain the standard curve after fitting it with the four-parameter Formula 1. The LOD and EC_{50} were calculated from the standard curve. The LOD value was obtained as the estimated antigen concentration, showing the mean blank fluorescence intensity plus three times the standard deviation (n = 3).

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$$y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^b} \quad (1)$$

4.7. Detection of Digoxin in Spiked Human Serum. To confirm that Q-body can be used to measure the concentration of digoxin in human serum, digoxin standard was added into human serum samples without digoxin at final concentrations of 1.5, 5.0, and 150 ng/mL. Serum samples were provided by the Affiliated Hospital of Weifang Medical University. PBST solution containing 20 ng/mL Q-body and another PBST containing 20% human serum sample were mixed in equal volume and incubated at 25 °C for 5 min. The fluorescence intensity was measured, and the concentration of digoxin in the measured samples was calculated by using the standard curve. The concentrations of digoxin in the measured serum sample were calculated finally.

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S.Z., R.Z., Y.G., Y.C., and S.Z.Z. carried out the experiments. S.Z., M.L., H.L., and J.D. wrote the manuscript draft. J.D. conceived the study and designed and supervised the experiments. All authors have reviewed and agreed to the final version of the paper.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Xia, X.; Cai, Y.; Cui, X.; Wu, R.; Liu, F.; Huang, K.; Yang, X.; Lu, X.; Wu, S.; Gu, D. Temporal trend in mortality of cardiovascular diseases and its contribution to life expectancy increase in China, 2013 to 2018. *Chin Med. J. (Engl)* **2022**, *135* (17), 2066−2075.

(2) Pashazadeh-Panahi, P.; Hasanzadeh, M. Digoxin as a glycosylated steroid-like therapeutic drug: Recent advances in the clinical pharmacology and bioassays of pharmaceutical compounds. *Biomed Pharmacother* **2020**, *123*, 109813.

(3) Varela-Chinchilla, C. D.; Sanchez-Mejia, D. E.; Trinidad-Calderon, P. A. Congenital Heart Disease: The State-of-the-Art on Its Pharmacological Therapeutics. *J. Cardiovasc Dev Dis* **2022**, *9* (7), 201.

(4) Vetticaden, S. J.; Chandrasekaran, A. Chromatography of cardiac glycosides. *J. Chromatogr.* **1990**, *531*, 215−34.

(5) Yang, H.; He, Q.; Eremin, S. A.; Pan, J.; Zou, Y.; Cui, X.; Zhao, S. Fluorescence polarization immunoassay for rapid determination of dehydroepiandrosterone in human urine. *Anal Bioanal Chem.* **2021**, *413* (17), 4459−4469.

(6) Dahal, U. P.; Jones, J. P.; Davis, J. A.; Rock, D. A. Small molecule quantification by liquid chromatography-mass spectrometry for metabolites of drugs and drug candidates. *Drug Metab. Dispos.* **2011**, *39* (12), 2355−60.

(7) Ooba, N.; Sente, A.; Abe, M.; Watanabe, F.; Tsutsumi, D.; Nakamura, K.; Nakayama, T.; Kimura, K.; Fukuoka, N. Frequency of Clinical Monitoring of Serum Concentrations of Digoxin, Potassium, and Creatinine, and Recording of Electrocardiograms in Digoxin-Treated Patients: A Japanese Claims Database Analysis. *Biol. Pharm. Bull.* **2020**, *43* (5), 913−916.

(8) Zeegers, J. J.; Maas, A. H.; Willebrands, A. F.; Kruyswijk, H. H.; Jambroes, G. The radioimmunoassay of plasma-digoxin. *Clin. Chim. Acta* **1973**, *44* (1), 109−17.

(9) Abe, R.; Ohashi, H.; Iijima, I.; Ihara, M.; Takagi, H.; Hohsaka, T.; Ueda, H. "Quenchbodies": quench-based antibody probes that show antigen-dependent fluorescence. *J. Am. Chem. Soc.* **2011**, *133* (43), 17386−94.

(10) Abe, R.; Jeong, H. J.; Arakawa, D.; Dong, J.; Ohashi, H.; Kaigome, R.; Saiki, F.; Yamane, K.; Takagi, H.; Ueda, H. Ultra Q-bodies: quench-based antibody probes that utilize dye-dye interactions with enhanced antigen-dependent fluorescence. *Sci. Rep* **2014**, *4*, 4640.

(11) Liang, J.; Dong, H.; Wang, H.; Yi, Z.; Jiang, G.; Inagaki, T.; Gomez-Sanchez, C. E.; Dong, J.; Ueda, H. Creation of a quick and sensitive fluorescent immunosensor for detecting the mineralocorticoid steroid hormone aldosterone. *J. Steroid Biochem Mol. Biol.* **2022**, *221*, 106118.

(12) Liang, J.; Dong, H.; Xu, F.; Li, B.; Li, H.; Chen, L.; Li, M.; Liu, Y.; Jiang, G.; Dong, J. Isolation of a Monoclonal Antibody and its Derived Immunosensor for Rapid and Sensitive Detection of 17beta-Estradiol. *Front Bioeng Biotechnol* **2022**, *10*, 818983.

(13) Li, H.; Zhu, B.; Li, B.; Chen, L.; Ning, X.; Dong, H.; Liang, J.; Yang, X.; Dong, J.; Ueda, H. Isolation of a human SARS-CoV-2 neutralizing antibody from a synthetic phage library and its conversion to fluorescent biosensors. *Sci. Rep* **2022**, *12* (1), 15496.

(14) Huston, J. S.; Levinson, D.; Mudgett-Hunter, M.; Tai, M. S.; Novotny, J.; Margolies, M. N.; Ridge, R. J.; Bruccoleri, R. E.; Haber, E.; Crea, R.; et al. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli. *Proc. Natl. Acad. Sci. U. S. A.* **1988**, *85* (16), 5879−83.

(15) Lee, H.; Wilson, D.; Bunting, K. V.; Kotecha, D.; Jackson, T. Repurposing digoxin for geroprotection in patients with frailty and multimorbidity. *Ageing Res. Rev.* **2023**, *86*, 101860.

(16) Charfi, R.; Ben Sassi, M.; Gaies, E.; Jebabli, N.; Daghfous, R.; Trabelsi, S. Digoxin therapeutic drug monitoring: age influence and adverse events. *Tunis Med.* **2020**, *98* (1), 35−40.

(17) Steiness, E. Suppression of renal excretion of digoxin in hypokalemic patients. *Clin Pharmacol Ther* **1978**, *23* (5), 511−4.

(18) Cummings, E. D.; Swoboda, H. D. Digoxin Toxicity. In *StatPearls*; StatPearls Publishing: Treasure Island, FL, 2022.

(19) Patocka, J.; Nepovimova, E.; Wu, W.; Kuca, K. Digoxin: Pharmacology and toxicology-A review. *Environ. Toxicol Pharmacol* **2020**, *79*, 103400.
(20) Ballotari, M.; Taus, F.; Tolle, G.; Danese, E.; Dorizzi, R. M.; Tagliaro, F.; Gottardo, R. Development of a new ultra-high-performance liquid chromatography-tandem mass spectrometry method for the determination of digoxin and digitoxin in plasma: Comparison with a clinical immunoassay. *Electrophoresis* **2022**, *43* (9−10), 1019−1026.
(21) Wheeler, M. J. Immunoassay techniques. *Methods Mol. Biol.* **2006**, *324*, 1−23.
(22) Embree, L.; McErlane, K. M. Comparison of digoxin analysis by high-performance liquid chromatography/post-column derivatization and fluorescence polarization immunoassay. *Xenobiotica* **1990**, *20* (6), 635−43.
(23) Kwong, E.; Embree, L.; McErlane, K. M. Comparison of analysis of digoxin in cardiac patients by HPLC and RIA. *Res. Commun. Chem. Pathol Pharmacol* **1990**, *68* (1), 121−124.
(24) Tada, A.; Rodrigues-Silva, C.; Rath, S. On-line solid phase extraction-ultra-high performance liquid chromatography coupled to tandem mass spectrometry for the determination of N-nitrosodiethanolamine in baby shampoo. *J. Pharm. Biomed Anal* **2021**, *202*, 114132.
(25) Seraissol, P.; Lanot, T.; Baklouti, S.; Mane, C.; Ruiz, S.; Lavit, M.; De Riols, P.; Garrigues, J. C.; Gandia, P. Evaluation of 4 quantification methods for monitoring 16 antibiotics and 1 beta-lactamase inhibitor in human serum by high-performance liquid chromatography with tandem mass spectrometry detection. *J. Pharm. Biomed Anal* **2022**, *219*, 114900.
(26) Li, H.; Sorensen, J. V.; Gothelf, K. V. Quantitative Detection of Digoxin in Plasma Using Small-Molecule Immunoassay in a Recyclable Gravity-Driven Microfluidic Chip. *Adv. Sci. (Weinh)* **2019**, *6* (6), 1802051.
(27) Kjelstrup, M. V.; Nielsen, L. D. F.; Hansen-Bruhn, M.; Gothelf, K. V. A DNA-Based Assay for Digoxin Detection. *Biosensors (Basel)* **2018**, *8* (1), 19.
(28) Bradbury, D. W.; Trinh, J. T.; Ryan, M. J.; Chen, K. J.; Battikha, A. A.; Wu, B. M.; Kamei, D. T. Combination of the lateral-flow immunoassay with multicolor gold nanorod etching for the semi-quantitative detection of digoxin. *Analyst* **2022**, *147* (18), 4000−4007.
(29) Gupta, R.; Gupta, P.; Wang, S.; Melnykov, A.; Jiang, Q.; Seth, A.; Wang, Z.; Morrissey, J. J.; George, I.; Gandra, S.; Sinha, P.; Storch, G. A.; Parikh, B. A.; Genin, G. M.; Singamaneni, S. Ultrasensitive lateral-flow assays via plasmonically active antibody-conjugated fluorescent nanoparticles. *Nat. Biomed Eng.* **2023**, DOI: 10.1038/s41551-022-01001-1.
(30) Tong, H.; Cao, C.; You, M.; Han, S.; Liu, Z.; Xiao, Y.; He, W.; Liu, C.; Peng, P.; Xue, Z.; Gong, Y.; Yao, C.; Xu, F. Artificial intelligence-assisted colorimetric lateral flow immunoassay for sensitive and quantitative detection of COVID-19 neutralizing antibody. *Biosens Bioelectron* **2022**, *213*, 114449.
(31) Bessette, P. H.; Aslund, F.; Beckwith, J.; Georgiou, G. Efficient folding of proteins with multiple disulfide bonds in the Escherichia coli cytoplasm. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96* (24), 13703−8.
(32) Levy, R.; Weiss, R.; Chen, G.; Iverson, B. L.; Georgiou, G. Production of correctly folded Fab antibody fragment in the cytoplasm of Escherichia coli trxB gor mutants via the coexpression of molecular chaperones. *Protein Expr Purif* **2001**, *23* (2), 338−47.
(33) Burks, E. A.; Chen, G.; Georgiou, G.; Iverson, B. L. In vitro scanning saturation mutagenesis of an antibody binding pocket. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94* (2), 412−7.

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