



Rapid detection of *mecA* gene of methicillin-resistant *Staphylococcus aureus* by a novel, label-free real-time capacitive biosensor

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ABSTRACT

This work presents a rapid, selective and sensitive automated sequential injection flow system with a capacitive biosensor for detection of the *mecA* gene (the model chosen for this study), which emerges from methicillin-resistant *Staphylococcus aureus*. A DNA-based 25-mer capture probe was immobilized on the surface of a gold electrode which was integrated in the capacitive sensor system. A constant current pulse was applied and the resulting capacitance was measured. Injection of the target DNA sample to the sensor surface induced hybridization to occur between the target and the complementary sequence, which resulted in a shift in the measured capacitance (ΔC). The ΔC was directly proportional to the concentrations of the applied target probe with linearity ranging from 10^{-12} to 10^{-7} M. The biosensor had a detection limit of 6.0×10^{-13} M and a recovery of 95 % of the *mecA* gene when spiked in human saliva. The biosensor showed a promising selectivity. It could clearly discriminate single-base, two-base and twelve-base mismatch probes with a decrease in the signal strength by 13 %, 26 %, and 89 %, respectively relative to the signal strength of the complementary target probe. There was no significant signal observed for the non-complementary probe. The biosensor-chip could be re-used for more than 12 cycles with residual capacity of 94.5 ± 4.3 % and a RSD of 4.6 % by regenerating the biosensor-chip with a solution of 50 mM NaOH.

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1. Introduction

Multiresistant microorganisms constitute a severe threat to modern healthcare. There is a need for development of sensitive and quick analyses. To develop such a system, focus was on identifying methicillin-resistance in *Staphylococcus aureus* (MRSA). *S. aureus* is causing several medical problems and it is also known that MRSA is causing hospital acquired infections [1].

Focus for developing new, sensitive analyses has so far been on the healthcare direct needs. However, it now becomes important to monitor spreading of these resistance genes in the environment, and as a subsequent result to monitor possible spreading of resistance genes to other organisms in the environment.

The fate of resistance genes needs to be monitored from the sick patient via wastewater to wastewater treatment plants, the sludge generated at the treatment plants and later on after spreading sludge as fertilized on farmland [2].

The spread of MRSA throughout the world differs, but according to The European Antimicrobial Resistance Surveillance System, the number of clinical isolates with MRSA increases. For example, the prevalence of MRSA in clinical isolates is only < 1 % in Northern Europe but > 40 % in southern Europe, > 50 % in the USA, 77 % in Taiwan and 64 % in South Korea [3]. The situation has further increased in severity during 2008–2013 according to WHO's report [4] where the overall reported range of resistance exceed 20 % in most WHO regions reported and even exceeds 80 % in some reports.

Resistance genes are coding for enzymes that will eliminate the effects of antibiotics.

MRSA is resistant to all β -lactam antibiotics, including penicillin, cephalosporins cloxacillin and methicillin.

Laboratory screening for resistance genes faces a serious challenge with regard to the currently available screening methods. Hence, there is a clear disproportion between sensitivity, turnaround time, selectivity and cost of the existing methods. Currently, e.g. MRSA-screening methods are categorized into culturing-based and molecular-based methods. The former is employed for phenotypic test, while the latter is for genotypic characterization. The culture-based methods comprise broth-

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plate- and chromogenic- media tests, which determine the antibiotics bacteriostatic or bacteriocidal effect to the microbe, in this case *S. aureus* [5]. Such methods are laborious and time-consuming, including up to 3 days incubation time [6]. The most common molecular methods that are used for e.g. MRSA-analysis are the polymerase chain reaction (PCR) and microarray technologies [5,7,8]. These methods are indeed sensitive and quick [8], but also significantly more expensive, hence unaffordable for many health care, educational and research institutions, mainly in developing countries.

Considering the background outlined above, priority in clinical researches must be set towards the development of tools for fast and cheap MRSA diagnosis. In this work, a novel, rapid and sensitive technique, i.e. a capacitive DNA-sensor, is presented for the detection of the *mecA* gene in spiked human saliva. The capacitive DNA-sensor registers hybridization of the target sequence of *mecA* gene to the capture DNA probe that is immobilized on the sensor chip. The instrumentation, CapSense™ Biosystem employs a current pulse of constant amplitude that is applied to the sensor electrode from which the registered capacitance is recorded. The principle and applications of capacitive transduction in DNA-sensors have previously been reported elsewhere ([9,10]) The hybridization of the target *mecA* gene to the capture probe results in a decrease in the registered capacitance, which is directly proportional to the concentration of the target.

Monitoring for multiresistant microorganisms has been focused on the medical sector, but recently it was also highlighted that the multiresistance genes might be spread via wastewater and from the wastewater treatment plants as a biofertilizer is spread on the farming fields [11].

2. Materials and methods

2.1. Materials

A 25-mer *mecA* gene probe was designed from *S. aureus* subsp. *aureus* MRSA252 *mecA* gene sequence, retrieved from the National Center for Biotechnology Information (NCBI) database [12] as shown in Table 1. The probes were purchased from Integrated DNA Technologies, Inc. (Leuven, Belgium). Human saliva was collected from one of the authors of this work. Absolute ethanol and sodium hydroxide were obtained from VWR international (Radnor, USA). Tyramine (99 %), acetone, *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl) *N*-ethylcarbodiimide hydrochloride (EDC), 6-mercaptohexanol (MCH), and 1-dodecane thiol were obtained from Sigma-Aldrich (Steinheim, Germany). All other chemicals used were of analytical grade. All buffers and regeneration solutions were prepared in ultrapure water (Millipore purification system, Massachusetts, USA). All solutions were filtered through a membrane (pore size 0.22 μm) and degassed prior to use.

Table 1
DNA probes used in this study.

Name of probe	Sequence (5'→3') (25-mer)
Capture probe(CP)	GCTCAGGTA ¹ CTGCTATCCACCCTCA
Complementary probe (TP)	TGAGGGTGGATAGCAGTACCTGAGC
Single-base mismatched probed (SMT)	TGAGGGTGGATG ¹ CAGTACCTGAGC
Two-base mismatched (TMT)	TGAGGGTGGAT ¹ GCAGTACCTGAGC
Twelve-base mismatched probe (12 M T)	TCACGCTCGIT ¹ TGAGAGCAGTGG
Non-complementary probe (NC)	ACTCCACCTAT ¹ CTCATGGACTCG

2.2. Methods

2.2.1. Fabrication of a sensor chip

A custom-made disposable electrode with a thin film of gold with a diameter of 3 mm and a gold purity of 99.99 % (Academic workshop, Linköping University, Sweden) was used as sensor chip in this study. The sensor chip was rinsed with ultrapure water and dried with a stream of nitrogen gas after each modification step. The electrodes were initially cleaned in acetone, followed by ethanol and finally Piranha solution (3:1 concentrated sulfuric acid: 30 % hydrogen peroxide), for 5 min in each solution. Thereafter, the electrodes were coated with a polytyramine (Pty) film by electropolymerization of tyramine on the electrode surface using cyclic voltammetry (CV), as explained elsewhere [13,14]. The capture probe was immobilized by covering the electrode surface with 10 μL of 10 μM capture probe (in 10 mM potassium phosphate buffer pH 7.2, containing 5 mM EDC, and 8 mM NHS). The electrode was left at room temperature for 2 h in a container with nitrogen atmosphere. This method was previously described by Teeeparuksapun et al. [13] with minor modification. The capture probe (ssDNA) is covalently immobilized on the electrode surface via chemical reactions between phosphate group on the capture probe and amine group of the polytyramine on the electrode surface, resulting in the formation of phosphoramidate bond. After immobilization, the electrode was immersed in 1-dodecanethiol (10 mM in ethanol) for 20 min in order to block pinholes on the affinity surface [9,10], and thereafter finally stored at 4 °C until use.

2.2.2. Experimental set up and current pulse for capacitance measurements

In this study, the principle of detection is based on the electrical double layer theory. The current pulse-capacitance measurements were performed in an automated sequential-injection system with software control developed by CapSense Biosystems AB (Lund, Sweden). A schematic diagram of the automated sequential-injection flow system is represented in Fig. 1.

The system consists of a centris pump, which is linked to a three-port valve and a five mL syringe pump. Port #1 is used to remove the waste during initialization while port #2 is connected to the supply of the working buffer. The syringe pump sucks buffer from the buffer container and dispenses into an injection loop via port #3. The injection loop is linked to a nine port valve. Ports #1 to #7 of the latter valve are used either for regeneration or infusion of standard and sample solutions. Port #8 is used for draining the waste. Port #9 is connected to a degasser unit, which in turn is connected to a three electrodes-flow cell [15]. A fixed sample volume of 250 μL is sequentially and automatically introduced into the three electrodes-flow cell, which is electrically connected to the capacitance measurement unit. This unit is equipped with an analog-to digital converter (ADC) unit and controlled by the in-house made software. A constant current pulse is automatically sent to the sensor chip, and the system automatically computes the total capacitance (C_{TOT}) over the sensor chip/solution interface. The C_{TOT} , also known as the electrical double layer (EDL), is calculated by Eq. 1 [15].

$$V = i(R_s + \frac{t}{C_{TOT}}) \quad (1)$$

where V is the total potential registered in the system after applying the current i during time t and with R_s being the ohmic resistance of the solution.

By plotting V vs. t , the linear curve is established, with V -intercept (V_0) = iR_s . Then C_{TOT} can be determined from the slope (u) of the linear curve.

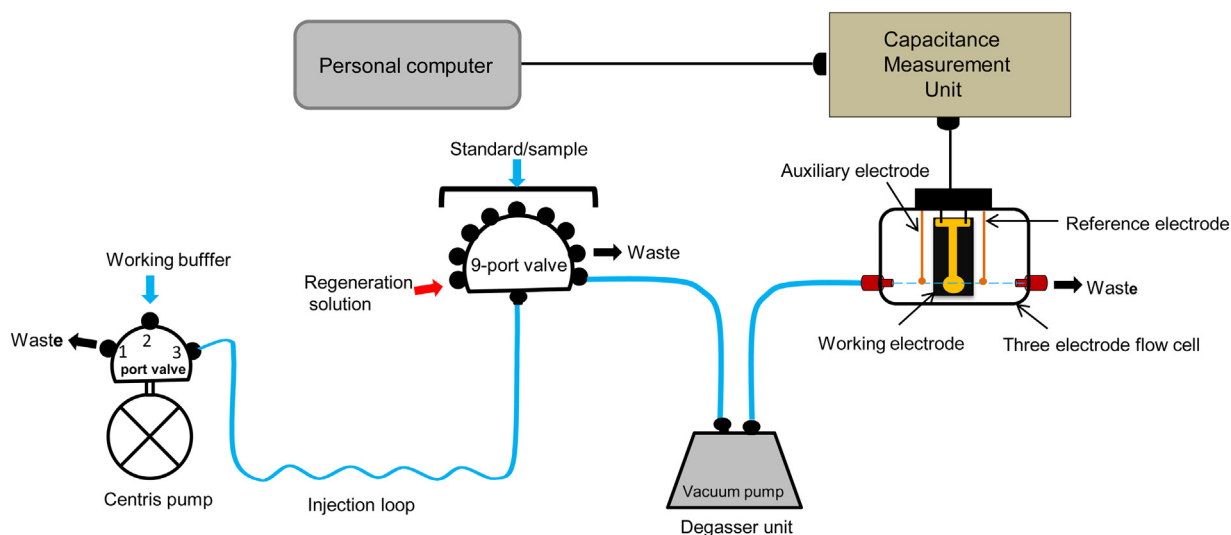


Fig. 1. Schematic illustration of an automated sequential injection flow system.

Since,

$$C_{TOT} = \frac{dQ}{dV_C} = \frac{idt}{dV_C} = \frac{i}{u} \quad (2)$$

where Q and V_C are the charge accumulated and the built-in potential across the EDL, respectively and u is the slope of the linear curve.

2.2.3. Establishment of a calibration curve at room temperature, 23 °C

Samples of the target probe were prepared in 10 mM potassium phosphate buffer

pH 7.2 at concentrations ranging from 10^{-6} to 10^{-14} M having a dilution factor of 10. The standard samples were placed in connection to the injection ports of the CapSense system for analysis. Each sample was analysed in triplicates, and the mean of

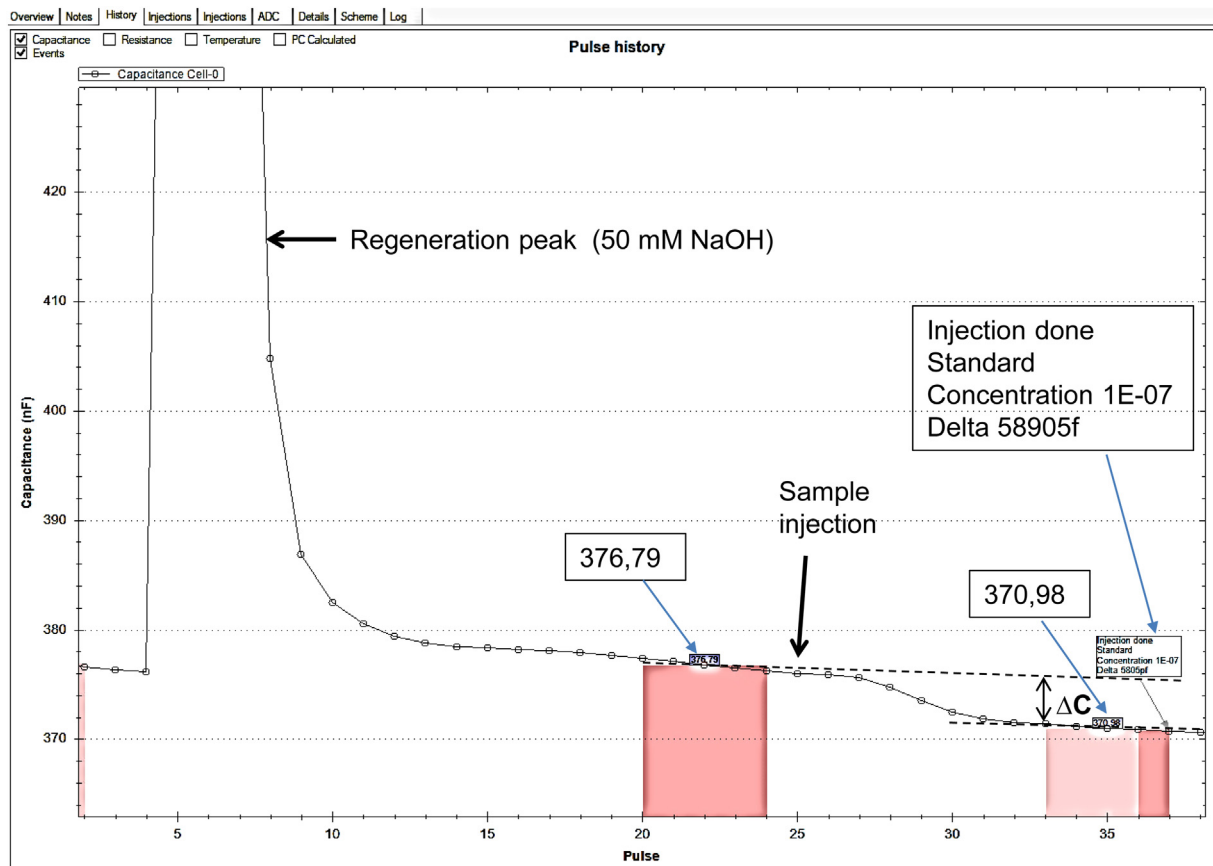


Fig. 2. Time course graph showing a signal (ΔC) after injection of target sample into the CapSense™ Biosystem. The time from injection to analysis was 10 min.

the three signal values with its standard deviation for each sample was calculated.

2.2.4. Re-usability of the sensor chip hosting the capture probe

Reusability of the sensor chip was determined by repeatedly injecting pulses of 10^{-9} M of the standard target probe with intermittent regeneration steps for 20 cycles, visualized in terms of the relative standard deviation (% RSD). The study was performed under the same conditions as that described for the establishment of the calibration curve.

2.2.5. Detection of base-mismatches

10^{-8} M of single-, two-, and twelve-base mismatches, as well as non-complementary probes in phosphate buffer with 30 % formamide were applied to the capacitive sensor system. The obtained signals for each probe were compared with that obtained for the standard target complementary at the same concentration.

2.2.6. Determination of matrix effects on the *mecA* gene

The 25-mer *mecA* gene standard samples were added to human saliva to make concentrations of 10^3 , 10, and 1.0 nmol L^{-1} respectively. The samples were then diluted by factors of 10^5 , 10^3 , and 10^2 in PB, respectively, so as to achieve the same final concentration of 0.01 nmol L^{-1} of the target *mecA* gene for analytical and comparative purposes. The % recovery of the *mecA* gene from each diluted spiked-saliva sample was determined by comparing a registered signal due to that sample with that one generated by non-spiked saliva sample at the same dilution factor (Eq. 3).

$$\% \text{ Recovery} = \frac{\Delta C_1 - \Delta C_2}{\Delta C_3} \times 100 \quad (3)$$

ΔC_1 and ΔC_2 are the signals of the *mecA* spiked saliva sample and non-spiked saliva sample at the same dilution factor, respectively; ΔC_3 is the signal of 0.01 nmol L^{-1} *mecA* gene in 10 mM phosphate buffer (PB) pH 7.2, registered from the calibration curve.

3. Results and discussion

3.1. Calibration and operational characteristics

Injection of the standard complementary target 25-mer *mecA* gene sample into the capacitive DNA-sensor system induced the hybridization reaction between injected *mecA* gene and the capture probe on the sensor chip, which resulted in a capacitance change (ΔC) as shown on Fig. 2.

The ΔC was directly proportional to the concentration of the injected target *mecA* gene sample. The curve of applied

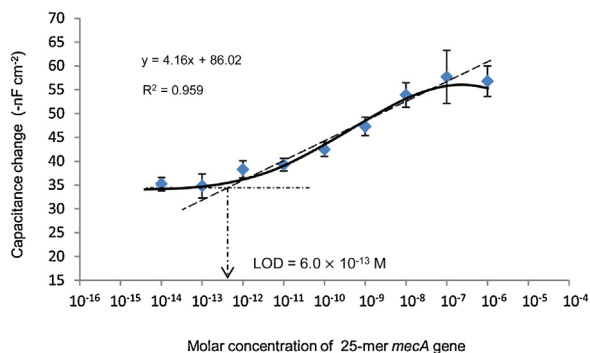


Fig. 3. The established calibration curve of the capacitive DNA-sensor showing a linearity range and limit of detection (LOD) for 25-mer *mecA* gene sample in PB. The assays were done at 23 °C.

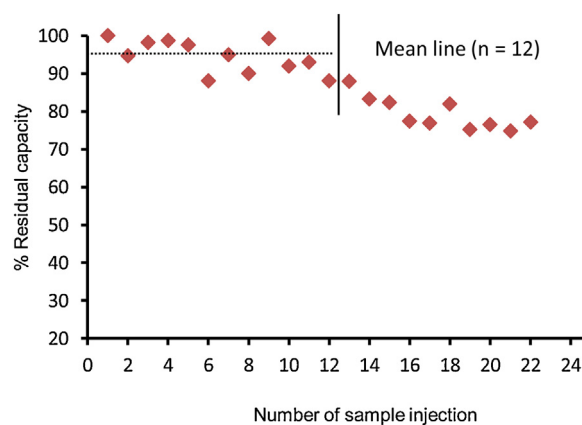


Fig. 4. Reusability of the sensor chip. 250 μL of a 10^{-9} M target *mecA* gene was repeatedly applied on the sensor chip up to 22 times with regeneration step between each individual assays.

concentrations vs. ΔC is represented in Fig. 3. A linear relationship was observed from 1.0×10^{-12} to 1.0×10^{-7} M, and the limit of detection (LOD) that was calculated according to Buck and Lindner [16], was found to be 6.0×10^{-13} M (0.6 pM). To the best of our knowledge this is the lowest reported LOD for nucleotide based analyses based on label-free techniques. The achieved LOD for the capacitive DNA-sensor was 40 and 100 times lower than values reported for electrochemical biosensors by Liu et al. [17] and Watanabe et al. [18], who reported LODs for the detection of *mecA* gene as low as 23 pM and 10 pM, respectively, utilizing a sandwich hybridization assay with the dual labelling technique, involving gold nanoparticles and alkaline phosphatase. For the 10 mM potassium phosphate buffer that was used in this work, the thickness of the diffuse layer was said to be less than 10 nm ($0.01 \mu\text{m}$). This implies that the counter ions (electrical double layer) were very close to the electrode surface [9,10], hence, making the proposed capacitive DNA-sensor to respond to extremely low concentration of the analyte.

The electrode was regenerated with pulses of 50 mM NaOH and could be used for more than 12 cycles with residual capacity of $94.5 \pm 4.3 \%$, and RSD value of 4.6 % as shown in Fig. 4.

3.2. Detection of base-mismatches

The ability of the developed capacitive DNA-sensor to detect different base-mismatches was investigated. Equal molar concentrations of target *mecA* gene samples, containing different base-mismatches were hybridized on the capture probe, and ΔC

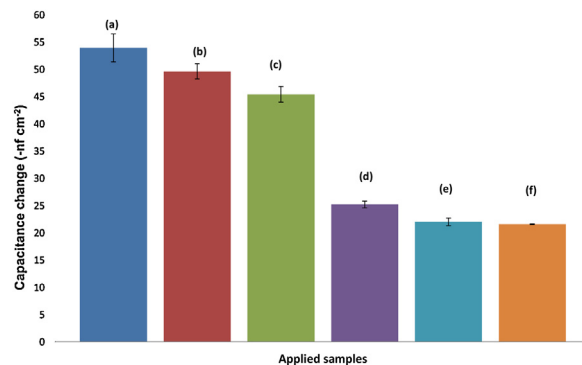


Fig. 5. Comparison of recorded signal upon hybridization of (a) complementary target (b) single-base-mismatch probe (c) two-base-mismatch probe (d) 12-base-mismatch probe (e) non-complementary probe to capture probe and (f) buffer alone.

Table 2
Determination of *mecA* gene in spiked saliva sample by capacitive DNA-sensor.

Sample dilution	Final concentration (nmol L ⁻¹)	Calculated concentration (nmol L ⁻¹)	Recovery (%)	RSD (n = 3) (%)
10 ²	0.01	0.0071	71	9.0
10 ³	0.01	0.0076	76	6.5
10 ⁵	0.01	0.0095	95	3.5

responses were recorded. A significant difference in signal (ΔC) between complementary target, single-base-mismatch, two-base-mismatch, and twelve-base-mismatch probes was observed (Fig. 5).

The signal strength was found to decline by 13 % and 26 %, for single-base and two-base, respectively, comparative to the complementary probe signal. The observed reduction in signal for mismatching probes is an effect of the base-pair break of the *mecA*-capture probe/*mecA* target duplex. It reveals the ability of the capacitive DNA-sensor to distinguish between complementary and other targets with base-mismatches. Insignificant signal, below starting capacity change, ($\Delta C < 35$ -nF/cm⁻²) was observed for a twelve-base mismatch- and a non-complementary-probes; this signal was the same as that observed for the buffer alone (22 ± 0.1 -nF/cm⁻²), which could be due to an effect of built-up back pressure (noise) released during sample injection.

3.3. Recovery of the *mecA* gene from human saliva

The *mecA*-spiked samples were analysed in triplicate and the average signals were used to calculate the percentage recovery of the target gene from human saliva. The results from the study are shown in Table 2. The highest percentage recovery with the lowest uncertainty was obtained in the most diluted sample, suggesting that further sample dilution decreases the effects of the matrix. However, the system needs to be optimized since too large sample dilution will lead to loss of signal amplitude. It should be noted that in this study the concentration of the analyte was kept constant while diluting the matrix.

4. Conclusion

This work has described a novel, automated, selective and sensitive fast biosensor technique for quantification of methicillin-resistant *S. aureus* based on the detection of the *mecA* gene. The developed biosensor could, with high sensitivity detect the *mecA* gene from the complex human saliva with a recovery of 95 %. The biosensor showed a remarkable selectivity, with the possibility to clearly discriminate the complementary probe, from single-base, two-base, twelve-base mismatch and non-complementary probes. This work provides a foundation to extend the study towards the analysis of real MRSA-*mecA* gene in clinical and environmental samples.

Declaration of Competing Interest

The authors do not have any conflict of interest.

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