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Binding strategies for capturing and growing *Escherichia coli* on surfaces of biosensing devices

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ABSTRACT

Antibiotic resistant bacteria have become a threat to world health. An advanced method of detection, based on matrix assisted laser desorption ionization time-of-flight mass spectroscopy can identify bacteria relatively rapidly, but it is not suitable to measure bacterial antibiotic resistance. Biosensors may be able to detect resistance by monitoring growth after capture on sensor surfaces but this option has not been addressed adequately. We have evaluated the growth of *Escherichia coli* after capture in 96 well microplates and observed that growth/capture efficiency was relatively similar for antibody-based techniques, but non-specific capture varied considerably. We confirm that neutravidin binds *E. coli* non-specifically, which limited its use with biotinylated antibodies or aptamers. Centrifugation enhanced bacterial growth/capture considerably, indicating that procedures enhancing the interaction between bacteria and surface-bound antibody have the potential to improve growth efficiency. Capture and growth required larger numbers of bacteria than capture and detection on biosensor surfaces. Previously, we reported that the minimum concentration of live *E. coli* required for initiating growth on a GaAs/AlGaAs biosensor was ~ 10^5 CFU/mL (Nazemi et al., 2018), and we speculated that this could be related to the poisonous effect of Ga- and As-ions released during dark corrosion of the biosensor, however in the present report we observed that the same minimum concentration of *E. coli* was required for growth in an ELISA plate. Thus, we argue that this limitation was related rather to bacterial inhibition by the capture antibodies. Indeed, antibodies at titres designed to capture bacteria inhibited bacterial growth when the bacteria were added to growth medium at titres less than 10^5 CFU/mL, indicating that antibodies may be responsible for the higher limits of sensitivity due to their potential to restrict bacterial growth. However, we did not observe *E. coli* release after 6 h following the capture indicating that these bacteria did not degrade antibodies.

1. Introduction

The rise in multi-resistant microorganisms represents a major threat for public health worldwide [1–3]. Microorganisms also cause problems for water, food, and pharmaceutical industries [3–6]. Current methods of microbial detection, like culture and detection in an automated instrument, polymerase chain reaction (PCR) and immunology-based methods such as enzyme-linked immunosorbent assays (ELISA), are time consuming (24–48 h), complex, require trained personnel and may require enrichment steps to detect low concentrations [7,8]. Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) has emerged as a promising method for rapid, sensitive and cost-effective bacterial identification, but provides only limited determination of antibiotic resistance [9,10]. Even though these techniques are reliable, the recent increase in antibiotic resistance of bacteria [1–3,11,12] has pushed health organizations to request alternative, fast, specific and sensitive methods for detection, identification and determination of antibiotic resistance of bacteria [13]. Biosensors [14] have been increasing in popularity as an alternative method due to their simplicity, speed, sensitivity, real-time monitoring, portability of the device and, potentially, low cost of testing [7,8]. Among numerous types of biosensors, the most frequently investigated appear to be those based on optical [15–17] and electrochemical [7,8] methods. Surface plasmon resonance (SPR)-based and electrochemical impedance-based sensors using gold (Au) surfaces have shown attractive sensitivity for bacterial detection [7,15], but have rarely been used to measure

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antibiotic sensitivity [18]. Photoluminescence-based biosensors that usually employ GaAs surfaces have been used for detection of some bacteria [19–24] allowing quantification down to 10^3 colony forming units of bacteria (CFU) per mL and even lower with chemotaxis [25]. Photoluminescence-based biosensors have also been employed to determine antibiotic resistance of *E. coli*, but only with relatively low limits of detection [24]. The response of these biosensors depends on the efficiency of capturing target biomolecules with target-specific ligands [26], such as antibodies [8,17], aptamers [27], antimicrobial peptides [28], lectins [15], phages [16] or molecular imprints [29] as opposed to non-specific binding to Au surface after coating with polylysine [18].

The present study focuses on determining the best capture efficiency that can be employed on a biosensor surface and understanding why capture and growth is so much less efficient than capture and detection. The subsequent growth of the captured bacteria is an important factor to consider in order to monitor bacterial growth for antibiotic sensitivity tests in further studies. Typically, Au-based surfaces have been employed by numerous biosensors, although a large variety of biosensors employ GaAs, Si, and oxide coated surfaces. The purpose of this work was to investigate different bacterial capture strategies while working with plastic surfaces of 96 well plates, which allowed testing different binding structures in a short period of time. To this end, we have investigated passively adsorbed goat and chicken antibodies that are the recognized reference method for antigen capture on ELISA plates [30]. We have compared them with strategies more amenable to GaAs or Au surfaces such as goat antibodies covalently linked to a plate with a carboxyl group and biotinylated goat antibodies or biotinylated aptamers linked via passively adsorbed neutravidin. Since low numbers of bacteria would not grow after capture, we employed high concentrations of bacteria as would be found in suspended bacterial colonies to be identified by MALDI-TOF. Although centrifugation is more challenging to input in a fully automated portable device, it can be considered as a strategy to investigate enhanced surface capture [31], e.g., as a proxy for other strategies such as chemotaxis or electrophoresis that might possibly overcome electrostatic repulsion between bacteria and surfaces. Centrifugation at less than 5000 G was not expected to disrupt surface integrity of bacteria [32] and it is easy to integrate into 96 well protocols. By growing bacteria captured by antibodies, we have investigated the ability of bacteria to degrade the antibodies and release themselves from capture.

2. Materials and methods

2.1. Materials and reagents

Tests were carried out with clear Corning 96 well plates 3590 (ELISA plate) from Fisher Scientific (Whitby, ON, CAN), and clear carboxylated 96 well plates (COOH plate) from BioWorld (Dublin, OH, USA). Common laboratory chemicals including NaH_2PO_4, Na_2HPO_4, NaCl, $NaHCO_3$, Na_2CO_3, polyoxyethylene sorbitan monolaurate (Tween-20), and $MgCl_2$ were from Fisher Scientific (Fair Lawn, NJ, USA). Bovine serum albumin (BSA) was from Roche Diagnostics (Indianapolis, IN, USA), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were from Sigma-Aldrich (St. Louis, MO, USA) or alternatively, EDC and NHS were from Biacore (GE Healthcare bio-science AB, Uppsala, Sweden). The purified IgG fraction of goat antibodies (Ab) directed against *E. coli*, both biotinylated and non-biotinylated, were from ViroStat (Portland, ME, USA), chicken Ab directed against *E. coli* from Immune Biosolutions (Sherbrooke, QC, CAN), biotinylated aptamers (Apt) (E2, EcO3R and EcO4R) [33,34] were synthesized by Integrated DNA Technologies (IDT) (Coralville, IA, USA), neutravidin (NA), Salmon sperm DNA and Tris-HCl were from Invitrogen (Burlington, ON, CAN). Tryptone, nutrient broth (NB) and agar were from Becton, Dickinson and Company (BD) (Mississauga, ON, CAN) and glucose from J. T. Baker (Phillipsburg, NJ, USA).

A genetically modified strain of *E. coli* K12 BW25113 continually expressing the green fluorescent protein (GFP) was provided by Sébastien Rodrigue from the Département de Biologie of the Université de Sherbrooke, and referred to as *E. coli* GFP. The *E. coli* GFP was conserved at 4 °C on nutrient agar (NB + 15% agar) medium and refreshed each month. A fresh culture of *E. coli* GFP was used each day of tests, after incubation overnight (O/N) at 37 °C in NB medium. Tryptone glucose (1% tryptone, 1% glucose and 0.5% NaCl) (TG) was used for the capture tests to allow subsequent growth and reduce the fluorescent background compared with NB or Luria-Bertani medium. Fluorescence was measured using a CytoFluor Series 4000 (Applied Biosystems, Foster City, CA, USA) microplate reader with excitation at 485 nm and emission at 535 nm. Data were exported to Microsoft Excel and GraphPad Prism version 7.03 for Windows (GraphPad Software, La Jolla, California, USA, www.graphpad.com) for further analysis.

2.2. Standard curve for quantification of E. coli GFP

In order to quantify captured *E. coli* GFP, a standard curve was made with unbound *E. coli* GFP using 200 µL of known concentrations of *E. coli* GFP suspended in TG medium in a 96 well plate without Ab. Concentrations of $10^{1.2}$, $10^{2.2}$, $10^{3.2}$, $10^{4.2}$, $10^{5.2}$ and $10^{6.2}$ colony forming units per mL (CFU/mL) were monitored for 12 h with data taken each 15 or 30 min in the CytoFluor. Data points for each growth curve were plotted to identify the time when fluorescence increased above background levels, which was called the Time threshold (Tt).

2.3. Passive capture of antibodies

For the capture of bacteria with Ab, 100 µL of goat or chicken Ab (concentrations tested included: 0; 0.5; 1; 2; 5; 10; 20; 50; 100 and 200 µg/mL) in carbonate buffer pH 9.6 were added to the wells of a 96 well plate and incubated O/N at 4 °C with agitation. The wells were washed 5 times with 300 µL of PBS + 0.05% Tween-20 (PBST) and 200 µL of blocking solution (PBST + 3% BSA) were added to the wells and incubated O/N at 4 °C with agitation.

2.4. Active capture of goat Ab with EDC/NHS

To test similar structures to those used with Au or GaAs surfaces functionalized with carboxyl-thiol linkers [35], COOH plates were used with activators EDC/NHS to covalently link the carboxyl group of the wells with an amine group from the Ab. For this structure, 50 µL of fresh solutions of 800 mM EDC in water and 200 mM NHS in water were used to activate the carboxyl group in the wells for 1 h at room temperature (RT). The EDC/NHS from Sigma-Aldrich and EDC/NHS from Biacore were tested separately for comparison. After washing the wells, 100 µL of Ab (concentrations tested: 0; 20; 100 and 200 µg/mL) in water were added and then incubated for 1 h at RT. The wells were washed between each step with PBST and blocked as mentioned above.

2.5. Capture of biotinylated antibodies or aptamers with neutravidin bound to surfaces

In order to simulate capture of biotinylated linkers to Au or GaAs surfaces (functionalized with biotin alkane thiols and NA) [20], we bound NA directly to 96 well plates. 100 µL of NA in PBS (concentrations tested: 0; 0.1; 0.2; 0.3; 0.4; 0.5; 0.6; 0.7; 0.8; 0.9; 1; 2; 3; 4; 5; 10; 20; 50; 100 and 200 µg/mL) was added to the wells and incubated O/N at 4 °C with agitation. The wells were washed and, for the antibody before bacteria (AB) method, 100 µL of biotinylated Ab (concentrations tested: 0; 20; 100 and 200 µg/mL) in PBS or 100 µL of biotinylated aptamer in their respective binding buffers (BB) (BB E2: PBST + 1% BSA + 0.1 mg/mL salmon sperm DNA, BB EcO3R and EcO4R: 10 mM Tris-HCl pH 7.5 + 0.5 M NaCl + 1 mM $MgCl_2$) [32,33] were added to

the wells and incubated 1 h at 37 °C with agitation. The washing and blocking steps were done as mentioned above.

Alternatively, in the pre-incubation (PI) method, biotinylated Ab or Apt were pre-incubated with 100 µL of $10^{8.5}$ CFU/mL of *E. coli* GFP in PBS or in the specific BB of each Apt in micro centrifuge tubes and incubated 1 h at 37 °C with agitation. The tubes were centrifuged 15 min at 2900 G and the supernatant was removed and the pellet washed with 100 µL of PBS or with the respective BB of each Apt. The content of the tubes was then transferred to the wells of the NA-coated plate and incubated 1 h at 37 °C with agitation. The blocking step was done as mentioned above and the steps of monitoring and analysis were performed as described below.

2.6. Capture of E. coli GFP with ligands bound to 96 well plates

After the blocking step, the wells were washed 5 times with PBST, then 200 µL of *E. coli* GFP (usually at $10^{8.2}$ CFU/mL, unless otherwise mentioned) in PBS was added to the wells and incubated 1 h at 37 °C with agitation. The wells were washed 5 times with 300 µL of PBST including 100 mM EDTA prior to addition of 200 µL of TG medium to the wells. The plate was incubated in a CytoFluor microplate reader for 12 h at 37 °C with fluorescence intensity measured (λ_{abs} = 485 nm, λ_{em} = 535 nm) every 15 or 30 min. A quantification curve with unbound *E. coli* GFP was constructed in parallel with each capture test. The quantity of bacteria captured was estimated by comparison with the parallel standard curve.

2.7. Centrifugation

Using the protocol described for passively adsorbed goat Ab, different concentrations of bacteria ($10^{0.2}$, $10^{1.2}$, $10^{2.2}$, $10^{3.2}$, $10^{4.2}$, $10^{5.2}$, $10^{6.2}$, $10^{7.2}$ and $10^{8.2}$ bacteria/mL) were added to the wells after the Ab adsorption and blocking steps. The plates were centrifuged 5 min at 250 G or 10 min at 550 G to pellet the bacteria gently [36] followed by incubation for 1 h at 37 °C to allow the Ab bound to the plate to capture the bacteria. The plates were then washed with PBST + 100 mM EDTA and incubated in the fluorometer as described above.

2.8. Antibody inhibition of bacterial growth

In order to titrate Ab inhibition of *E. coli* GFP growth, 100 µL of goat anti-*E. coli* Ab at concentrations of 0.5; 1; 2; 5; 10; 20; 50 and 100 µg/mL in TG and 100 µL of *E. coli* GFP (concentrations tested: $10^{0.5}$, $10^{1.5}$, $10^{2.5}$, $10^{3.5}$, $10^{4.5}$, $10^{5.5}$ and $10^{6.5}$ CFU/mL) in TG were added to wells of a 96 well plate. The plate was incubated in the fluorometer with data taken every 15 min as above.

2.9. E. coli GFP degradation of captured antibodies

In order to ascertain if *E. coli* GFP could degrade Ab in the well after capture, we have investigated the growth of bacteria at 37 °C in TG medium in uncoated and Ab functionalized wells. The number of bacteria was determined after washing designed to remove Ab released bacteria. *E. coli* GFP was captured on plates prepared with passively adsorbed goat Ab and unbound bacteria washed off as described above leaving attached bacteria in the well with 200 µL of TG. The plate was incubated in the fluorometer. After 0–6 h, some wells of the plate were washed with PBST + 100 mM EDTA and 200 µL of TG was added to the washed wells to quantify the bacteria still bound, and investigate if bacteria were still retained by the Ab or if the bacteria had degraded the Ab and been removed by the washing step.

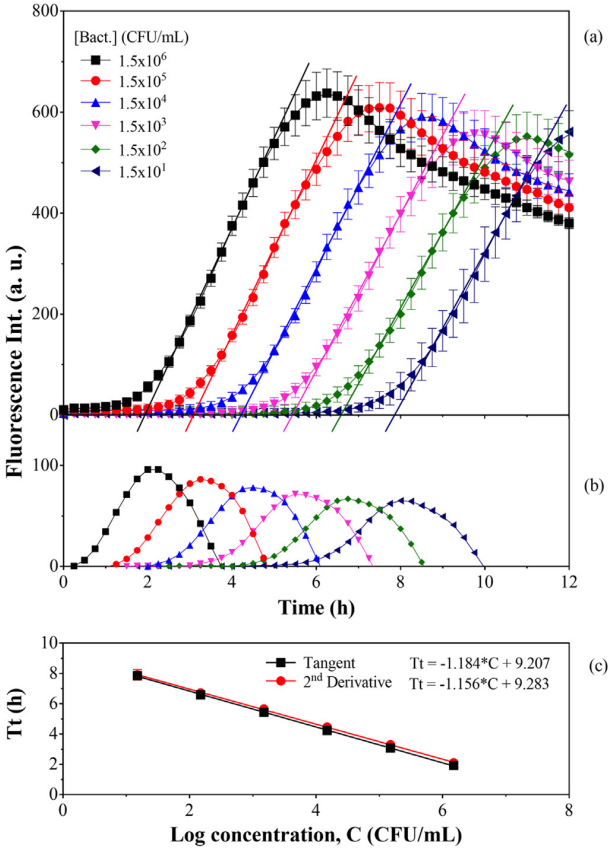

Fig. 1. Time dependent fluorescence intensity from different bacterial dilutions grown in TG in microtitre plate wells (a). Tangents of the ascending portion of the curves between 25% and 75% of maximal fluorescence were drawn to allow estimation of Tt from the intersection with the abscissa. Maxima of 2nd derivatives of the plots in Fig. 1a also allow Tt values to be estimated (b). Dependence of Tt on bacterial concentration (c).

3. Results

3.1. Quantification of E. coli GFP

Growth curves for *E. coli* GFP synthesis were analyzed to determine the time when fluorescence increased above background levels (Tt), either by identifying the abscissa crossing point of the tangent of the ascending portion of the curves between 25% and 75% of maximal fluorescence [37], as shown in Fig. 1a, or using the maximum of the second derivative of the growth curve [38], as shown in Fig. 1b. Standard plots of Tt versus bacterial concentration were constructed for both of these methods as shown in Fig. 1c. Initial concentrations of bacteria could be estimated from these plots by measuring the Tt of unknown samples.

3.2. Capture of E. coli with passively bound antibodies

As a standard reference binding interface, Ab were adsorbed to 96 well ELISA plates. The results, summarized in Table 1, indicate that *E. coli* GFP bacteria were captured with passively adsorbed goat Ab at $10^{4.3}$ CFU/mL when 10^{8} CFU/mL of these bacteria in PBS were added to wells. When chicken Ab were passively adsorbed, a comparable concentration of *E. coli* GFP at $10^{4.0}$ CFU/mL was captured. In the absence of Ab, *E. coli* GFP were non-specifically captured by the structure up to $10^{0.2}$ CFU/mL.

Table 1
Summary of capture methods.

Method	Specific (ligand-based) Log C_{sp} (CFU/mL)	Non-specific (no ligand) Log C_{nsp} (CFU/mL)	Specific - non specific Log (C_{sp}/C_{nsp})	[NA] (μg/mL)	[Ligand]
Passive Goat Ab	4.3 ± 0.8	0.1 ± 0.1	4.2	–	200 μg/mL Ab
Passive Chicken Ab	4.0	0.2 ± 0.4	3.8	–	unknown
Covalent Biacore	3.5 ± 1.4	0.0 ± 0.2	3.5	–	200 μg/mL Ab
Covalent Sigma	3.7 ± 1.1	2.2 ± 1.9	1.5	–	200 μg/mL Ab
NA-Antibody PI	4.9 ± 0.6	2.3 ± 0.7	2.6	1	100 μg/mL Ab
NA-Antibody AB	4.0 ± 0.6	2.8 ± 0.8	1.2	20	50 μg/mL Ab
NA-Aptamer PI	5.2 ± 0.7	3.3 ± 1.2	1.9	100	E2 500 nM
NA-AptamerAB	3.7 ± 0.7	3.3 ± 1.2	0.4	100	E2 500 nM

Passive Goat Ab = Reference method by using passively adsorbed goat Ab.
Passive Chicken Ab = Method using passively adsorbed chicken Ab.
Covalent Biacore = Method using COOH-plate and EDC/NHS from Biacore with goat Ab.
Covalent Sigma = Method using COOH-plate and EDC/NHS from Sigma with goat Ab.
NA-Antibody PI = Pre-incubation Method using passively adsorbed NA and biotinylated goat Ab.
NA-Antibody AB = Antibodies before bacteria Method using passively adsorbed NA and biotinylated goat Ab.
NA-Aptamer PI = Pre-incubation Method using passively adsorbed NA and biotinylated Apt.
NA-Aptamer AB = Antibodies before bacteria Method using passively adsorbed NA and biotinylated Apt.

3.3. Capture of *E. coli* with covalently bound goat IgG antibodies

Ab were linked to a COOH plate that had been activated with EDC/NHS to covalently link an amine group of the Ab. With EDC/NHS from Biacore, *E. coli* GFP were captured at $10^{3.5}$ CFU/mL with goat Ab. With EDC/NHS from Sigma-Aldrich a comparable ligand-based binding was achieved at $10^{3.7}$ CFU/mL, but this was also associated with a considerably more non-specific capture of bacteria at $10^{2.2}$ CFU/mL compared to EDC/NHS from Biacore giving 10^{0} CFU/mL (Table 1). The number of bacteria captured by the covalently bound Ab increased over 10-fold for the 4-fold increased concentration of Ab solutions (from 5 to 200 μg/mL), which is comparable to the increased number of bacteria captured on passively bound Ab for the same increase of the concentration of Abs (Table 2).

3.4. Capture of *E. coli* with biotinylated antibodies and neutravidin

Neutravidin was bound passively to an ELISA plate, then biotinylated Ab followed by bacteria (AB method) or bacteria preincubated with biotinylated Ab (PI method) were added. More efficient bacterial capture was observed with the PI method than with the AB method ($10^{4.9}$ CFU/mL versus $10^{4.0}$ CFU/mL). *E. coli* GFP were non-specifically captured at $10^{2.3}$ CFU/mL with 1 μg/mL of NA passively adsorbed on an ELISA plate without Ab (Table 1). The log difference between the specifically and non-specifically captured bacteria was 2.6 for NA employed to capture biotinylated Ab-bacteria complexes (PI method) versus 1.2 with the AB method.

Table 2
Capture of bacteria by different amounts of passively adsorbed or covalently-bound antibodies.

Ab conc. (μg/mL)	Passively adsorbed Ab Log C (CFU/mL)	Covalently bound Ab Log C (CFU/mL)
200	3.9 ± 0.3	3.7 ± 1.1
100	3.8 ± 0.3	3.5 ± 1.2
50	3.7 ± 0.3	3.2 ± 1.3
20	3.4 ± 0.4	2.9 ± 1.3
10	3.4 ± 0.5	2.8 ± 1.4
5	3.0 ± 0.4	2.6 ± 1.4
1	1.6 ± 0.5	2.0 ± 1.3
0.5	1.1 ± 0.6	2.4 ± 2.0
0	0.3 ± 0.5	2.2 ± 1.9

* Different concentrations of Ab were passively adsorbed or covalently bound to surfaces. Capture of *E. coli* was with PBST for the Passively Adsorbed Ab method and with PBS for the Covalently-Bound AB method. Sigma EDC/NHS was used for the Covalently-Bound AB method.

3.5. Capture of *E. coli* with biotinylated aptamers and neutravidin

Neutravidin was also used to capture biotinylated Apt either before bacterial capture (AB method) or as aptamer-bacteria complexes (PI method). The AB method did not show an increase over non-specific binding with NA (Table 1). With the PI method, *E. coli* GFP were captured at $10^{5.2}$ CFU/mL, versus $10^{3.3}$ CFU/mL observed for non-specifically captured bacteria with NA and without added aptamer.

3.6. Increasing capture efficiency with centrifugation

It is assumed that bacterial capture on surfaces is hindered by electrostatic or other repulsive forces. Several strategies have been proposed to overcome this repulsion and improve contact with the surface such as centrifugation, electrophoresis and chemotaxis. We attempted to improve capture by centrifuging bacteria onto the surface. With the addition of centrifugation at 250 G for 5 min, $10^{7.0}$ CFU/mL *E. coli* GFP were captured from suspensions containing $10^{8.2}$ CFU/mL compared to $10^{4.3}$ CFU/mL captured without centrifugation (Table 3). When centrifugation was performed at 550 G for 10 min, $10^{7.1}$ CFU/mL of *E. coli* GFP were captured. However, centrifugation also gave rise to higher amounts of non-specifically captured bacteria (on surfaces without Ab added) at $10^{4.1}$ CFU/mL with centrifugation at 250 and 550 G (Table 3).

When capture was attempted on surfaces with passively absorbed goat Ab, without centrifugation, bacteria were captured at $10^{4.3}$ and $10^{3.4}$ CFU/mL from $10^{8.2}$ and $10^{7.2}$ CFU/mL suspensions, respectively, but no bacteria were captured from a $10^{6.2}$ CFU/mL suspension. When centrifugation was employed at 250 G, bacteria were captured from suspensions containing $10^{8.2}$ CFU/mL ($10^{7.0}$ CFU/mL), $10^{7.2}$ CFU/mL ($10^{6.0}$ CFU/mL) and $10^{6.2}$ CFU/mL ($10^{4.1}$ CFU/mL), and it also allowed detection down to $10^{5.2}$ CFU/mL ($10^{2.2}$ CFU/mL) (Table 3). Similar results were observed with centrifugation at 550 G.

3.7. Inhibition of bacterial growth by antibodies

Tests showed that there was minimal ($10^{2.2}$ CFU/mL) bacterial capture when $10^{5.2}$ CFU/mL or fewer bacteria were added to wells for capture with centrifugation, and none when fewer than $10^{7.2}$ CFU/mL were added without centrifugation. In order to determine whether Ab could have contributed to this lower capture efficiency by inhibiting bacterial growth, different concentrations of bacteria were added to wells with different concentrations of Ab. Fig. 2 demonstrates the effect

Table 3
Use of centrifugation to enhance capture of *E. coli* GFP on ELISA plates without Ab and with passively absorbed goat Ab.

Centrifugation[a]	Log Bact. Conc. (CFU/mL)	Captured bacteria		Bacteria
		With Ab Log C (CFU/mL)	Without Ab Log C (CFU/mL)	Net capture Log (C_{Ab}/C_{nAb})
No	8.2	4.3 ± 0.8	0.1 ± 0.1	4.2
	7.2	3.4	0	3.4
	6.2	0	0	0
250 G 5 min	8.2	7.0 ± 0.5	4.1 ± 0.7	2.9
	7.2	6.0 ± 0.1		
	6.2	4.1 ± 0.3		
	5.2	2.2 ± 0.4	1.5	0.7
	4.2	0		
550 G 10 min	8.2	7.1 ± 1.0	4.1 ± 0.1	3.0
	7.2	6.5		
	6.2	4.8		
	5.2	2.2 ± 1.0	0.2	2.0
	4.2	0		

[a] Bacteria at different concentrations were added to wells with or without passively adsorbed goat anti-*E. coli* Ab using 20 μg/mL of Ab. The plates were centrifuged at 250 G for 5 min or 550 G for 10 min, or not centrifuged, followed by 1 h incubation at 37 °C, and then bacteria titrated from the growth curves.

of growth inhibition of bacteria by the increasing amount of Ab. Suppression of bacterial growth was much more evident with 25 and 50 μg/mL than with 10 μg/mL or less of Ab, and particularly for bacterial concentrations of 1.5×10^4 or less.

3.8. Bacterial degradation of antibodies and release from plates

In Fig. 3, we compared the number of bacteria captured in uncoated wells of a microtitre plate and in wells coated with Ab at 100 and 20 μg/mL. The number of bacteria that remained bound to those wells after up to 6 h of incubation in TG was measured following washing to remove released bacteria. A slightly greater concentration of bacteria was observed captured with Ab at 100 μg/mL, compared with Ab at 20 μg/mL, while a negligible number of bacteria was captured in wells without Ab. However, it can be seen that regardless of whether Ab at 20 μg/mL or 100 μg/mL had been employed, and despite washing, the number of retained bacteria increased with the incubation time, indicating capture of progeny rather than release due to Ab degradation. Clearly, under conditions of the current experiment, we could conclude that *E. coli* GFP did not degrade the Ab that captured it.

4. Discussion

We have quantified bacteria capable of growing after capture on surfaces by measuring the doubling time necessary to reach the minimum number of bacteria required to produce detectable GFP fluorescence (Fig. 1). This method resembles the strategy used for the quantitative polymerase chain reaction (qPCR) where DNA copies double until they reach a minimal detectable amount called the quantification cycle threshold (Ct). This is usually ascertained by identifying the first maximum value from the second derivative of the fluorescence curve [38,39]. Because this value may depend on amplification efficiency, it was proposed to identify the abscissa crossing point of the tangent of the inflection point of the Richard's curve obtained by non-linear regression of the raw fluorescence data [37]. In our quantification method with *E. coli* GFP, the bacteria also doubled each 20–30 min and the Tt can be determined by this method as well. As it can be seen in Fig. 1c, both the second derivative fluorescence data and data obtained from the abscissa crossing points provided a comparable dependence of Tt on bacterial concentration.

Our method is optimal for bacteria expressing fluorescent products like *E. coli* GFP, but methods based on optical density determination would be necessary for non-fluorescent bacteria. Interference with contaminating bacteria from the environment, however, could affect results, especially when few target bacteria are captured. When we followed *E. coli* growth with optical density at 600 nm, our observed Tt were similar (data not shown). The qPCR technique could also be employed to quantify bacterial capture, but it would be more expensive and would probably not be able to quantify small numbers of bacteria captured as accurately as our procedure due to qPCR inherent lack of sensitivity with fewer than 10 bacteria [40–42] and with the requirement to use only part of the bacterial DNA extract from a well in small PCR reaction volumes.

Our procedure to quantify live *E. coli* based on growth is not as rapid as qPCR detection for low numbers of bacteria. Our experiments showed that the minimum concentration of live *E. coli* required for detection within 3 h was at $10^{5.2}$ CFU/mL with single bacteria detection requiring as much as 9 h (Fig. 1).

It was striking that the number of input bacteria able to grow after capture on an ELISA plate by a passively adsorbed goat Ab decreased precipitously from $10^{3.4}$ to 0 when the number of bacteria exposed to a surface decreased by only one order ($10^{7.2}$ to $10^{6.2}$) (see Table 3 for data with no centrifugation). This implies that capture with growth does not proceed with one-hit kinetics, or alternatively, that growth is inhibited in a non-linear fashion. As results documenting capture and detection without growth have indicated linear relationships between number of bacteria captured and number presented [7,35,43], it could be assumed that capture occurs by one-hit kinetics and probably implies that capture occurs when one Ab molecule interacts with one bacteria, and does not require several Ab with variable-binding energy to reach a total binding energy sufficient to oppose disruptive forces like electrostatic repulsion. Inhibition of growth must be suspected as the explanation for the rapid decline in growth potential after capture. Previously, we observed that the growth of *E. coli* on GaAs or Au surfaces required a minimum of 10^5 CFU/mL [44], while testing the sensitivity of *E. coli* strains to antibiotics with a photoluminescence-based biosensor was not possible for bacterial concentrations under 2×10^8 CFU/mL [24]. It has been suggested that the toxic effects of Ga or As ions released by GaAs-based biochips might be the cause of this requirement for high bacterial concentration necessary to allow growth. In the experiments reported here, Ga and As were not present, and so inhibition may have resulted from the Ab used to capture the bacteria on the surface. Indeed, Ab added to cultures of bacteria inhibited bacterial growth dependent on the concentration of the Ab and the concentration of the bacteria. It might be possible to overcome this inhibition if it were possible to find an Ab that did not inhibit bacterial growth, but did capture the bacteria, in an analogous fashion to Ab that neutralize viruses versus Ab that react with viruses but do not neutralize them [45]. It is generally

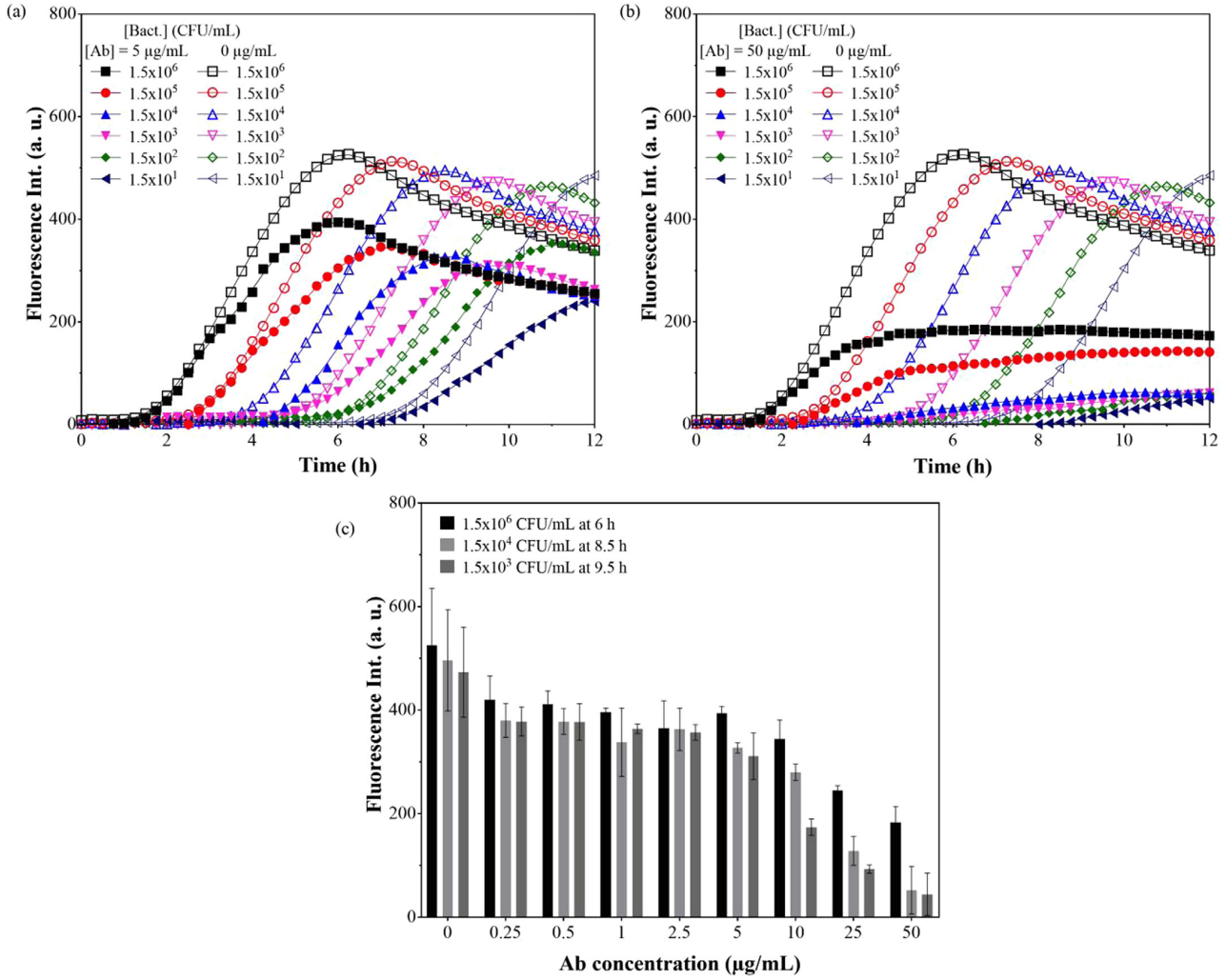

Fig. 2. Comparison of time dependent fluorescence intensity from *E. coli* GFP bacterial dilutions between 1.5 × 10 and 1.5 × 10^6 CFU/mL grown in TG in microtitre plate wells without anti-*E. coli* Ab, or in the presence of anti-*E. coli* Ab at 5 µg/mL (a) and 50 µg/mL (b), and as a function of anti-*E. coli* Ab concentration after 6, 8.5 and 9.5 h of incubation (c).

considered that antibodies classically inactivate bacteria in conjunction with complement or by opsonisation to enhance phagocytosis [46]. A few studies have shown direct antibody killing of bacteria by interaction with membrane bound enzymes [47,48] or lipopolysaccharides [46,49]. Chicken IgY antibodies have also been shown to directly kill *E. coli*. [50].

We employed centrifugation to overcome static repulsion between surfaces and bacteria and increase bacterial contact with Ab. Centrifugation at low speed was used so as not to affect the bacterial surface [32]. Chemotaxis and electrophoresis have also been shown to improve capture of bacteria, e.g., *E. coli* and *Legionella pneumophila* by attracting them to glucose generated at the surface from lactose cleavage by surface-bound galactosidase enzyme [25]. Other authors have brought bacteria into contact with a surface by electrophoresis [51] or using alternating current electrokinetic effects [52]. These methods have been shown to improve the efficiency of capture and merit further study to improve and simplify them.

Purified IgG goat and IgY chicken Ab bound passively to ELISA microplates gave similar crude growth/capture rates (Table 1). When goat Ab were bound covalently to microplates approximately 3–5 times fewer bacteria grew after capture than when using passively bound Ab. The lowest background values were observed with the EDC/NHS chemistry activated MHDA manufactured by Biacore. When NA was used to capture biotinylated Ab or Apt, it was found to bind *E. coli* resulting in high background levels for wells without Ab as has been reported previously [53]. Other bacteria may have less interaction with NA as it has been demonstrated previously for *L. pneumophila* [21] and *Staphylococcus aureus* [54]. Preincubating biotinylated Ab or Apt with bacteria and then adding to wells with NA already bound resulted in better crude rates of growth/capture than when bacteria were added to wells with biotinylated Ab or Apt already bound to the wells with passively adsorbed NA. However, even with preincubation, growth/capture was still not as efficient as with passively or covalently bound Ab.

The diversity of the capture molecules tested in this study was limited to polyclonal goat, polyclonal chicken Ab and Apt, which act like monoclonal Ab. It could be interesting to compare the capture efficiency between polyclonal and monoclonal Ab. Some studies have shown efficiency of polyclonal or monoclonal Ab and even compared different structures [7,8,55], but none has compared the efficiency of polyclonal vs monoclonal Ab with the same structure. The use of Apt for capture and detection of bacteria has been demonstrated [32,56–58]. In

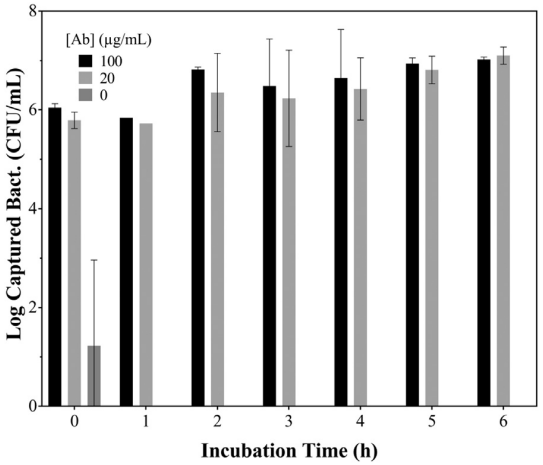

Fig. 3. Concentration of *E. coli* GFP bacteria captured in wells of a microtitre plate coated with Ab at 100 or 20 µg/mL, washed, and then incubated at 37 °C in TG for up to 6 h, followed by washing to remove released bacteria. Note that the concentration of bacteria captured on the uncoated surface of a well was negligible in comparison to that of bacteria captured on Ab coated well surfaces.

this study, Apt from other studies were used for the capture of *E. coli* GFP, but the results showed only modest efficiency due to high background.

Monitoring bacterial sensitivity to antibiotics requires that the bacteria be growing. The present study indicates that Ab used to capture bacteria might contribute to the high level of bacterial concentrations required for studying reactions to antibiotics. It might be possible to reduce the impact of Ab on bacterial growth by choosing monoclonal Ab that capture the bacteria by targeting epitopes that do not inhibit bacterial growth. Our study shows that *E. coli* K12 BW25113 didn't degrade the Ab used for capture – a feature potentially attractive for biosensors designed for investigating resistance of these and similar bacteria to antibiotics. Other types of bacteria, particularly *Streptococcus*, may be capable of specifically degrading Ab [59], or produce extracellular proteases that will degrade Ab. In the absence of reducing agents, however, Ab are relatively resistant to other proteases [60].

5. Conclusions

As a proxy for a biosensor surface, we have employed a 96 well ELISA plate surface in order to study different structures for binding and growth of bacteria. In comparison with reference passively adsorbed goat Ab, we have investigated passively adsorbed chicken Ab, covalently linked goat Ab to a plate with a carboxyl group and biotinylated goat Ab or biotinylated aptamers with passively adsorbed NA. With the GFP *E. coli* K12 BW25113, we found that passively bound goat or chicken Ab was a better approach for tethering bacteria and allowing growth than a covalent binding strategy. Our experiments showed that the minimum concentration of live *E. coli* required for detection within 3 h was at $10^{5.2}$ CFU/mL. Only $10^{3.4}$ CFU/mL of bacteria was captured on an ELISA plate with passively captured goat Ab from the initial concentration of bacteria at $10^{7.2}$ CFU/mL, which represented a ~ 10^{-4} fraction of the initial concentration. Furthermore, no capture of bacteria was observed when $10^{6.2}$ CFU/mL were added to the microplate. Adding a centrifugation step considerably increased the capture of bacteria, but it also increased the background contribution. The use of NA resulted in increased non-specific interactions, but better absolute capture performance and could be investigated further. Inhibition of bacterial growth with Ab in the growth medium could translate into inhibition of growth when capturing bacteria on a surface. In the presence of Ab at 10 µg/mL, we observed a drastic decrease of bacterial growth in TG medium at 1.5×10^5 CFU/mL and at lower bacterial concentrations. The growth of bacteria was only minimally affected if exposed to Ab at less than 10 µg/mL. It might be possible to overcome the Ab growth inhibition of bacteria, if it were possible to find an Ab analogous to those that are known to neutralize viruses versus Ab that react with viruses but do not neutralize them [45]. Of particular importance to the development of future Ab-based immunosensors of bacterial reactions to antibiotics is that we have not observed degradation of anti-*E. coli* K12 BW25113 Ab, even after 6 h of incubation.

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