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Engineering Biorthogonal Phage-based Nanobots for Ultrasensitive, *in situ* Bacteria Detection

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Abstract: Advances in synthetic biology, nanotechnology, and genetic engineering are allowing parallel advances in areas such as drug delivery and rapid diagnostics. While our current visions of nanobots may be far off, a generation of nanobots synthesized by engineering viruses is approaching. Such tools can be used to solve complex problems where current methods do not meet current demands. Assuring safe drinking water is crucial for minimizing the spread of waterborne illnesses. While extremely low levels of fecal contamination in drinking water are sufficient to cause a public health risk, it remains challenging to rapidly detect *E. coli*, the standard fecal indicator organism. Current methods sensitive enough to meet regulatory standards suffer from either prohibitively long incubation times or requirement of expensive, impractical equipment. Bacteriophages, tuned by billions of years of evolution to bind viable bacteria and readily engineered to produce custom proteins, are uniquely suited to bacterial detection. We have developed a biosensor platform based on magnetized phages encoding luminescent reporter

enzymes. This system utilizes bioorthongonally functionalized phages to enable site-specific conjugation to magnetic nanoparticles. The resulting phage-based nanobots, when combined with standard, portable field equipment, allow for detection of <10 cfu/100 ml of viable *E. coli* within 7 hours, faster than any methods published to date.

Introduction

The concept of nanobots has long intrigued scientists and engineers. Self-replicating nanoscale robots which can be used to repair, detect, deliver, or destroy bacteria, cancer cells, toxins or other similar, would be a technological revolution in science and medicine. While the popular depictions of mechanical nanobots are still in the distant future, scientists have proposed many versions of nanobots which can perform tasks such as deliver drugs[1,2], detect cancer[3], and move within cells[4]. The nanobots to date have typically been biological in nature, such as DNA structures, or inorganic such as nanocoils. The next generation of nanobots is possibly a hybrid of organic and inorganic components which can be put together to perform custom functions. These technologies can be used to provide tools for real-world problems for which the current technologies are limited.

The United Nations has declared safe drinking water a fundamental human right[5], yet fecal contamination affects the primary drinking water sources of over 2 billion people worldwide[6] and the associated pathogenic bacteria caused 502,000 deaths in 2012 alone[7]. Regulations mandate zero detectable colony forming units (cfu) of generic *E. coli* (an indicator of fecal contamination[8]) in 100 ml of drinking water[9]. Since fecal contamination may be undetectable by taste or smell, illnesses often spread *via* inadvertent consumption of contaminated water. Indeed, the World Health Organization estimates that assuring drinking water safety can reduce deaths from waterborne fecal bacterial pathogens by up to 73%[7]. Diagnostic assays giving observable signal

for low levels (<10 cfu/100 ml) of *E. coli* are therefore crucial in limiting the public health impact of contaminated drinking water.

Standard methods detect *E. coli* by culturing on selective media until visible colonies form (generally 24-48 hours)[10,11]. Though extremely sensitive (limit of detection <10 cfu/100 ml), the required multi-day incubation time translates to a slow response time and thus additional illnesses. This prolonged time to detection is due to rate-limiting step of colony formation. More recently developed biosensors promise faster detection[12–18] by shifting signal away from colony formation but often require equipment unsuitable for the low-resource settings where demand for these tests is highest[7]. Bacteriophage-based detection offers an unparalleled combination of sensitivity and speed,[19–28] leveraging the specificity of bacteriophages ("phages", bacteria-specific viruses) to their target bacteria[19–21,23,24,26,27,29–31]. Phage replication, which takes place on host cell machinery, involves precise regulation of viral gene expression levels. Phage-based detection takes advantage of their remarkable biorecognition and directed protein expression properties to create a unique platform for rapid, sensitive diagnostic assays[20–22,27,30,32–35].

Introducing genes with an observable product (e.g. fluorescent proteins) into the phage genome[21,22,27,32,33,35] creates a reporter phage that gives detectable signal during infection. Reporter phage-based assays have been reported to detect 1 cfu/100 ml in 10 hours[19]. The limiting step in reporter gene expression is phage infection, though this can take <30 minutes. Therefore, increasing the likelihood of infection by engineering concentration steps into reporter phage assays can reduce detection time further.

Immobilizing phages to solid supports (filters, nanoparticles, etc.) creates bacterial affinity materials that capture cells from solution for detection in a concentrated volume[23,28,29]. Phage orientation is crucial in these systems, though challenging to control. While phage capsids and tails

have similar chemistry, only phages conjugated *via* their capsids retain infectivity. Conjugation techniques reliant on native phage chemistry cannot distinguish between capsids and tails, limiting system potency. While capsid biotinylation orients phages on avidin-decorated surfaces[17,22,28], the required reagents are cost-prohibitive for water testing in low-resource areas. Phages represent an ideal nanobot scaffold due to their ability to bind specific bacteria, replicate, and be genetically engineered.[32,36,37] The remarkable tunability of phage properties allows for precise control over structure and function.

In this paper, we present a novel water test integrating sensitive reporter phage technology with low-cost, site-specific immobilization. We use amber suppression and directed protein self-assembly to alkynylate a luminescent reporter phage[38] that covalently conjugates to azide-decorated magnetic nanoparticles[39] to afford a phage-based bioaffinity nanobot that, in combination with two concentration steps, detects <10 cfu *E. coli* in a 100 ml sample within 7 hours. By incorporating unnatural amino acids with unique conjugation abilities on phages, additional functionalities can be incorporated by conjugating enzymes or other moieties in the future.

Results & Discussion

Assay design

E. coli detection in drinking water requires a highly sensitive detection platform to meet the 1 cfu/100 ml regulatory requirement and to be practical for use in the low-resource areas most affected by poor water safety must be accomplished by an inexpensive, portable apparatus. The assay outlined in Figure 1 is engineered to achieve these disparate goals by employing a robust luminescent reporter phage (NRGp17, a variant of T4 expressing the reporter enzyme nanoluciferase[40] fused to a cellulose-binding module[27] under the native small outer capsid protein

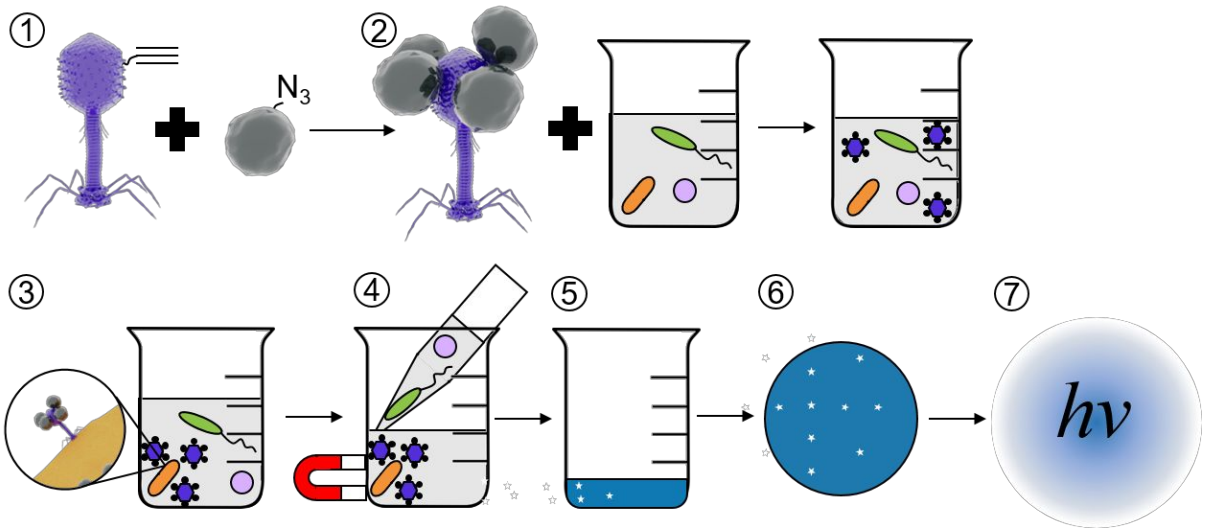

Figure 1: Schematic of *E. coli* detection assay. Self-assembled alkynylated T4 Nluc CBM phages are conjugated to azide-coated magnetic nanoparticles (MNPs) (1) using click chemistry. The resulting magnetic phage-based bioaffinity nanobot is added to samples of environmental water (2), where the phages bind specifically to *E. coli* (orange rods, 3). Beads, along with phages and *E. coli* are collected with a magnet and excess volume is aspirated (4). Infection cycle completes (5), releasing Nluc-CBM into solution. Samples are filtered (6) on cellulose to immobilize Nluc-CBM and luminescence is measured (7) to detect *E. coli*. Please note that the schematics in this figure are not to scale. T4 Nluc CBM is approximately 200 nm long and 900 nm in diameter. MNPs are approximately 15 nm in diameter(Table S9).

(SOC) promoter[30]) and multiple concentration steps with equipment already common in field testing. Luminescent intensity is inversely proportional to surface area, so we maximized sensitivity by concentrating the signal from the entire 100 ml into a single 0.05 cm^2 filter. To achieve this concentration using standard, portable field equipment, we employ two separate steps: (1) magnetic separation of target bacteria from solution and (2) vacuum filtration to immobilize the reporter enzyme fusion (Nluc cellulose binding module [CBM]) in the cellulose filter. Though we quantified luminescence in the lab using a spectrophotometer, previous research has demonstrated that luminescent signals can be readily captured using portable methods such as long-exposure photography using either a digital camera or smartphone[19,38]. Similarly, vacuum filtration is the current standard for portable water testing in developing countries.

Click T4 Nluc CBM Design

The described assay uses luminescent bacteriophages conjugated to superparamagnetic magnetic nanoparticles (MNPs) as the primary detection and concentration agent (Figure 1). To avoid

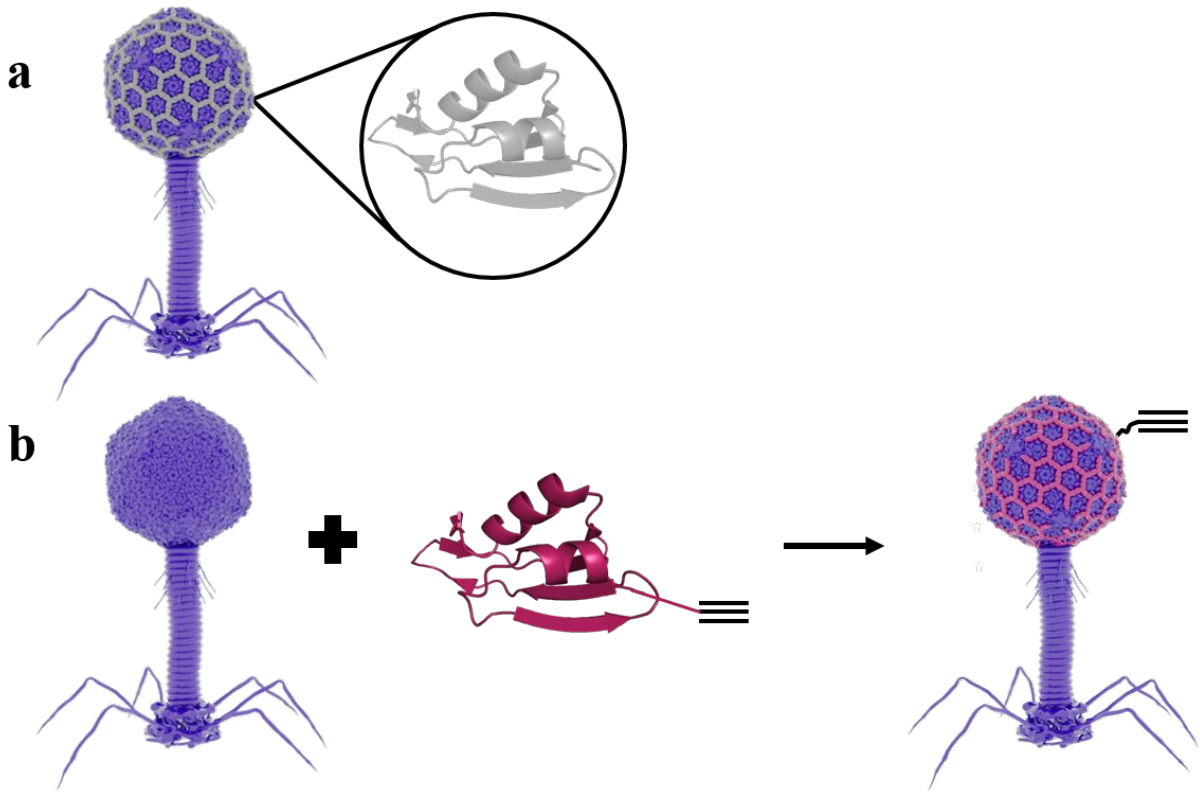

Figure 2: T4 phage capsid alkyne decoration strategy and Click SOC construct design. a) Schematic of WT SOC (grey, PDB ID 3IGE) assembly on the T4 capsid (PDB ID 5VF3). b) A SOC with a alkyne-containing unnatural amino acid is incorporated into the phage capsid to allow for click chemistry conjugation. Each phage capsid can accommodate 870 SOC proteins. Click-SOC self-assembled on T4 Nluc CBM (Δ*soc*). Click-SOC is shown in red with an alkyne schematic.

nonspecific interactions that minimize potency, we used Huisgen cycloaddition (click chemistry)[39] to form covalent bonds between alkynylated phages and azide-decorated MNPs. We functionalized the modified T4 phage NRGp17 with alkyne groups (Click NRGp17) by developing an alkynylated variant of the small outer capsid protein (SOC). This 9 kDa nonessential T4 protein self-assembles in a honeycomb pattern to form a homomeric 870-unit cage on the capsid[41–43] (Fig 2a). Previous research has shown that recombinant SOC can self-assemble on phage scaffolds with their native SOC deleted[41,42]. Phage display experiments have shown that SOC tolerates modification at both the N and C termini[43], and structural studies demonstrate that both termini are exposed to solution in the fully assembled cage[42]. Since NRGp17 is a SOC-knockout, we therefore

hypothesized that by incubating it with recombinant SOC alkynylated at the N-terminus, we could build phages that display up to 870 alkyne groups on their capsids (Figure 2b).

Click SOC design, expression, and analysis

Because no native amino acids contain alkyne functionality, we used the pEvol system[44] to engineer Click SOC, a SOC derivative containing propargylglyoxy phenylalanine (prp), an alkynyl derivative of tyrosine, at its N-terminus in addition to a C-terminal 6X polyhistidine tag for purification (Figure S1a,b). For the protein scaffold, we chose SOC from RB69, a phage closely related to T4. This protein is homologous to T4 SOC but expresses with higher yield in recombinant systems[42]. Previous research has shown that RB69 SOC has as high affinity for the T4 capsid as the native T4 SOC[42]. After recombinantly expressing Click SOC in BL21 (DE3) *E. coli* and purifying using immobilized metal affinity chromatography (Figure S2), we verified alkyne insertion by reacting the protein with 3-azido-7-hydroxycoumarin, a molecule that becomes

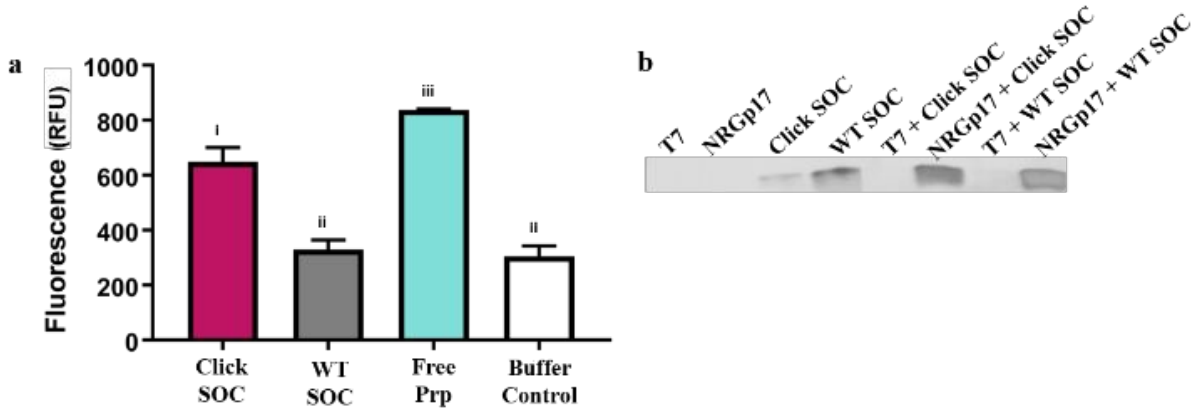

Figure 3: Click SOC has alkyne functionality and retains self-assembly properties. a) Fluorescence of 3-azido-7-hydroxycoumarin after click reaction. Click SOC has significantly higher fluorescence than WT SOC or the buffer control, indicating the successful introduction of available alkyne groups. Error bars indicate standard deviation of four technical replicates. Letters indicate significance by one-way ANOVA and Tukey's multiple comparison test ($p < 0.05$). b) Western blot against the C-terminal 6X His tag on WT SOC and Click SOC before and after self-assembly on phages confirms Click SOC has the same T4 self-assembly properties as WT SOC. T7 was used as a negative control for self-assembly. The raw data used to construct this figure are reported in Table S3.

fluorescent upon undergoing click cycloaddition[45] (Figure 3a). Click SOC was significantly more fluorescent in this experiment than WT SOC (649 ± 2.2 relative fluorescence units (rfu) vs. 329 ±

34.5 rfu, $p > 0.05$), indicating the presence of free alkyne groups. WT SOC was not significantly more fluorescent than a protein-free control, indicating that the alkyne group on Click SOC is specifically reacting with the azido fluorophore.

Directed self-assembly of Click SOC on NRGp17

The alkynylation of NRGp17 relies on previously demonstrated self-assembly of SOC on the phage capsid. To show that Click SOC has these same self-assembly properties, we incubated the purified protein with NRGp17, which was engineered as a *soc* deletion mutant. After centrifugation to pellet the phages, we performed a western blot against the C-terminal 6X polyhistidine tag (Figure 3b). Both WT SOC and Click SOC pelleted along with T4, showing self-assembly. Importantly, this pulldown behavior was not observed in samples of WT SOC or Click SOC incubated with T7, another icosahedral phage distantly related to T4, indicating that assembly is specific to the T4 capsid and not merely nonspecific adsorption to protein surfaces.

Synthesis of phage-based bioaffinity nanobots

Having shown that Click SOC is both alkynylated and able to self-assemble onto the T4 capsid, we next incorporated the magnetic component of the nanobot by using the click reaction[39] to conjugate Click NRGp17 to azide-decorated MNPs. After the reaction was complete, we magnetically captured the resulting phage-based bioaffinity nanobots and washed the resulting pellet with excess buffer. We found that upon resuspension, the nanobots had an infectivity of 5.10 ± 0.17 log plaques per ml of suspension (pfu/ml), while the final washes were only infective at 3.42 ± 0.10 log pfu/ml (Figure 4a). This significant difference in infectivity indicates oriented, immobilized phages, critical for the bacterial capture step of the assay in Figure 1. Furthermore, WT SOC gave a significantly lower yield of immobilized phages than Click SOC (4.00 ± 0.13 log

pfu/ml) (Figure 4b). This Click SOC-dependent activity retention demonstrates the stronger conjugation and site-specificity of the click conjugation as compared to any potential random adsorption of the WT SOC.

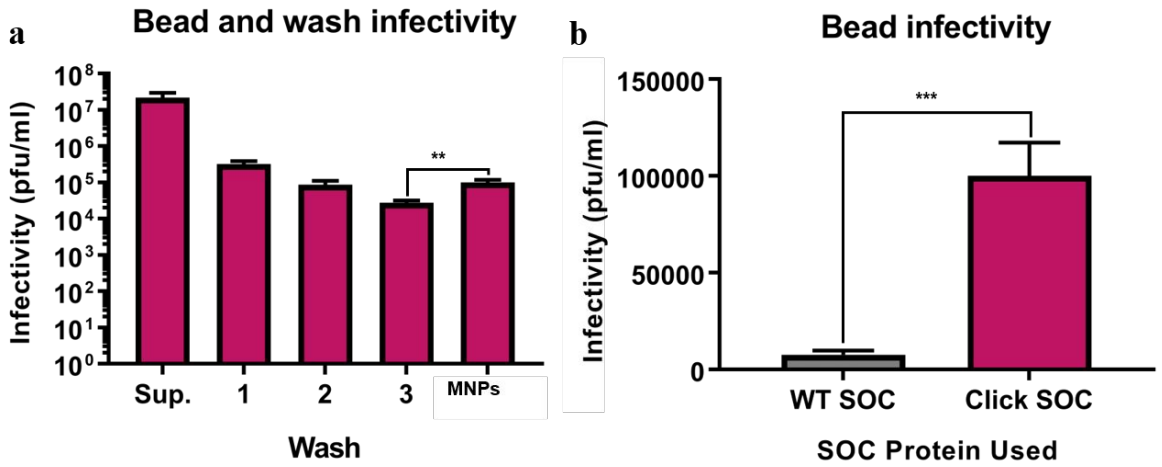

Figure 4: Immobilized phages retain infective properties after magnetic capture. a) The resuspended nanobots are significantly more infective than the final wash, indicating that phages have been magnetically captured and resuspended during the washing process. b) Infectivity of MNPs conjugated to NRGP17 by either WT SOC or Click SOC. Click SOC conjugation results in significantly higher infectivity, indicating a stronger conjugation between the phage and the bead. Error bars indicate standard deviation of three technical replicates. Asterisks indicate significance (** = $p < 0.01$, *** = $p < 0.001$) by unpaired two-tailed t-test. The raw data used to construct this figure are reported in Table S4.

Click conjugation of phages to MNPs creates sensitive nanobots

While the plaque assays showed that Click NRGp17 nanobots have significantly more infectivity than their WT SOC counterparts (Fig 4b), real-world challenge studies are necessary to demonstrate their high sensitivity and potential in a true assay setting. Therefore, we set up small (100 µl) samples containing 3.64 ± 0.32 log cfu/ml of *E. coli* (ECOR#13), an environmental *E. coli* isolate previously used in detection studies. The four detection reagents we tested (MNPs on their own, free NRGp17, WT NRGp17 nanobots, Click NRGp17 nanobots), gave luminescent signals of 3.5 ± 2.4, 74 ± 59, 75 ± 31, and 170 ± 100 relative luminescence units (rlu), respectively (Figure 5a). Importantly, only Click NRGp17 nanobots were sensitive enough to give luminescence above the baseline sterile water signal (Figure 5a). These data, in combination of the

plaque infectivity data, suggest that every component (phages, MNPs, Click SOC) is crucial to the sensitivity, supporting the scheme in Figure 1.

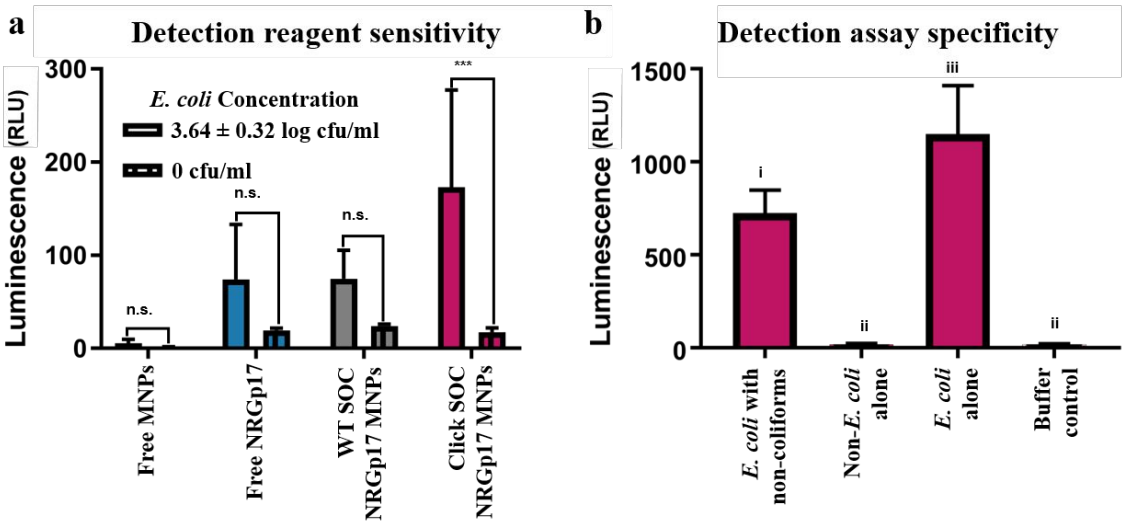

Figure 5: MNPs with click-immobilized NRGp17 are a specific, sensitive detection tool. a) Small-scale *E. coli* (ECOR#13) detection with no pre-enrichment. 100 µl samples were treated with 5 log ml^{-1} unconjugated azide-coated MNPs, 5 log pfu/ml free NRGp17, NRGp17 conjugated to MNPs via WT SOC, or Click NRGp17 conjugated to MNPs. Only the latter gives a signal significantly higher than the baseline luminescence. b) Small scale detection of 4.01 ± 0.15 log cfu/ml *E. coli* with no pre-enrichment in pure and mixed culture. Non-coliform bacteria included 3.27 ± 0.33 log cfu/ml *Pseudomonas aeruginosa*, 3.82 ± 0.22 log cfu/ml *Listeria monocytogenes*, and 3.23 ± 0.07 log cfu/ml *Sphingopyxis alaskiensis.* Error bars indicate standard deviation of four technical replicates. Bacterial concentration reported as mean ± standard deviation of 12 plate counts. Letters and stars indicate significance ($p < 0.05$; n.s. = $p > 0.05$; *** = $p < 0.001$) by one-way ANOVA and Tukey's multiple comparison test. Raw data used to construct this figure are reported in Tables S5 and S6.

Phage-based nanobots are specific to *E. coli*

To show that the Click NRGp17 nanobots are specific to *E. coli*, we prepared a cocktail containing 3-4 log cfu/ml of each of three non-*E. coli* bacterial strains (*Pseudomonas aeruginosa, Listeria monocytogenes, Sphingopyxis alaskiensis)*, all water-isolated or otherwise common in fresh water. The Click NRGp17 nanobots gave negligible (19 ± 4.5 rlu against a background of 19 ± 2.9 rlu) luminescent signal for these non-target strains, demonstrating their specificity to *E. coli* (Figure 5b). Furthermore, the nanobots gave significantly more luminescent signal (724 ± 124 rlu) for *E. coli* in mixed culture, demonstrating the high specificity of this assay to its target organism, with little interference by non-target environmental strains.

Magnetic nanobots detect <10 cfu *E. coli* in 100 ml within 7 hours

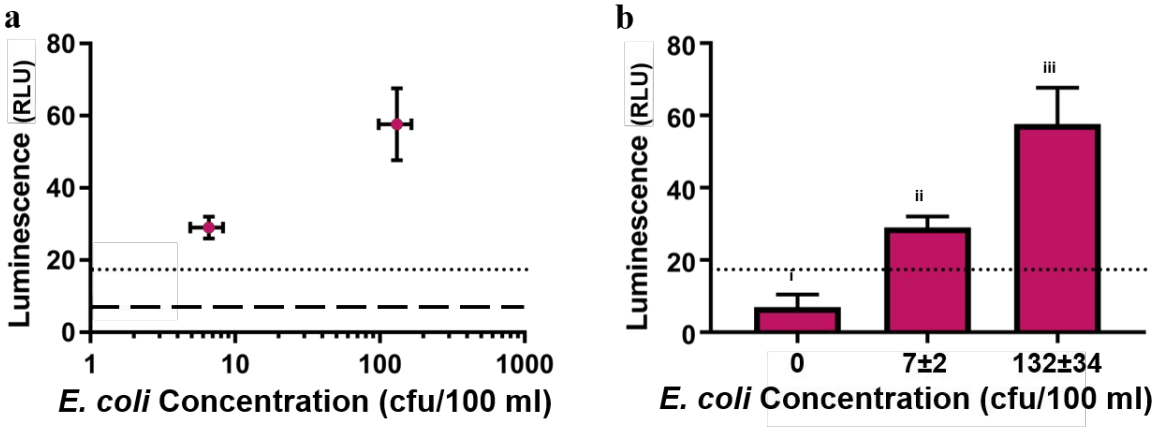

Figure 6: Phage-based bioaffinity nanobots can detect <10 cfu *E. coli* in 100 ml tap water within 7 hours. Luminescence error bars indicate standard deviation of 3 technical replicates. Dotted lines indicate LOD (3 standard deviations above mean baseline luminescence). a) *E. coli* (ECOR#13) concentration plotted against luminescence. Dashed line indicates baseline luminescence. Concentration error bars indicate standard deviation of 12 plate counts. b) Luminescence values obtained from different ECOR#13 concentrations. Letters indicate significance ($p < 0.05$) by one-way ANOVA and Tukey's multiple comparison test. Bacterial concentration reported as mean ± standard deviation of 12 plate counts. Raw data used to construct this figure are reported in Table S7.

Drinking water regulations require testing 100 ml sample[46] for *E. coli*, so we scaled up the detection platform to demonstrate performance at this volume. We prepared sterile tap water samples in 100 ml collection vials similar to those used in the field and inoculated them with known concentrations of *E. coli* (ECOR#13). To maximize the sensitivity of the assay, we pre-enriched the samples by adding concentrated media and incubating for 3 hours to allow injured bacteria to recover and allow all bacteria to approach the log growth phase. Since maximizing magnetic surface area minimizes collection time, we taped bar magnets to 10 cm square supports in the pattern shown in Figure S1c. These magnetic rigs have the same footprint as 10 cm square sterile petri dishes, which hold ~100 ml, thus making them ideal magnetic incubation vessels. We incubated the water samples for 10 minutes with the Click NRGp17 nanobots, captured in square petri dishes on the magnetic rigs for 10 minutes longer, and concentrated the samples 200-fold by resuspending captured bacteria in 500 µl LB media. The capture media was spiked with additional NRGp17 to ensure that all cells present were included in the luminescent signal. After allowing the infections to progress during a further 3-hour incubation, we vacuum filtered the samples

through single wells of a 384-well filter plate, which immobilized the Nluc CBM expressed by the phages into a 0.05 cm^2 area for detection using the NanoGlo kit. This assay gave a luminescent signal of 29 ± 3 rlu for 7 ± 2 cfu of *E. coli* in 100 ml against a background luminescence of 7 ± 3 rlu. The entire assay, from collecting water samples to reading luminescence, required less than 7 hours, which is less than 1/3 the time required for standard culture methods[11]. While it is impossible to reproducibly make samples containing exactly 1 cfu in 100 ml, we consistently detected <10 cfu in the same volume and the 16 rlu limit-of-detection (3 standard deviations above the baseline luminescence) was well below the 29 rlu observed luminescence for 7 ± 2 cfu, the lowest inoculated samples tested (Figure 6).

CONCLUSIONS

Testing drinking water for the presence of *E. coli* is an essential step in mitigating the spread of waterborne illnesses in the developing world. To comply with international and national regulatory standards, water tests must be able to detect 1 cfu of generic *E. coli* in 100 ml drinking water[46]. We developed an assay for *E. coli* contamination that detects <10 cfu in 100 ml drinking water within 7 hours, more than 3-fold faster than the standard EPA method[11] and 30% faster than previously published phage-based detection methods[19], and with demonstrated specificity in a cocktail of non-target organisms. It remains unknown if the assay can detect a single cfu given the practical limitations of preparing a sample containing a single cfu in 100 mL of water. This assay uses a combination of standard, portable field equipment (magnets, vacuum filtration, long-exposure photography) in combination with novel phage-based magnetic nanobots. Using amber suppression and directed self-assembly, we created a luminescent reporter bacteriophage with alkyne functionality. By conjugating this alkynylated phage to magnetic nanoparticles via the click reaction, we achieved spatial control of phage orientation on a solid surface without the need for

expensive reagents. The resulting phage-based magnetic nanobots are capable of binding bacteria, separating them from solution via magnetic capture, and detecting them via luminescence. By engineering a reagent with this level of polyfunctionality, we are able to achieve high sensitivity through multiple concentration steps.

Phage functionalization expands the potential utility of phages in bioengineering. Amber suppression technology is widely used for real-time visualization of biological processes, development of biocompatible materials, and spatial resolution in living systems[47]. The technique described in this study: production of recombinant phage-display proteins site-specifically tagged with artificial amino acids followed by directed self-assembly, is widely applicable beyond just the alkyne-tagged SOC reported. Given the range of artificial amino acids previously described in the literature[47], it is possible to use this technique to build phages with a wide range of functionality (e.g. photosensitivity, fluorescence, incorporation in polymers, etc.). Though amber suppression has been previously used in phage display[48], the method we used offers over 100-fold higher density of the artificial amino acid per capsid and allows for functionalization without the need for genetically modifying a potentially complex phage genome. Phages are widely used in biotechnology research and are an emerging antimicrobial and biocontrol agent[49], so the potential impact of facile introduction of novel functionality extends far beyond the water testing presented in this study. While this paper demonstrates the ability to detect low concentrations of *E. coli* (ECOR#13) from a large volume sample, the selection of different phage cocktails can allow the detection of numerous other bacterial indicators of pathogens.

MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. pEvol-pPrpRS[44], encoding propargylglyoxy phenylalanine (prp) RNA synthetase under an araBAD promoter, was a gift from Peter Schultz at the Scripps Research Institute (San Diego, CA, USA) and used unaltered. Sterile coliform test bottles were purchased from Corning Inc. (Corning, NY, USA). Multi-well filter plates (0.2 μm pore-size, nitrocellulose membranes) were purchased from Pall (Cortland, NY, USA). 4-20% gradient SDS-PAGE gels and nitrocellulose western blot membranes were purchased from BioRad (Hercules, CA, USA). Custom DNA oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA).

Bacterial and Phage Strains and Growth Conditions

All strains are described in Table S1. Unless otherwise noted, *E. coli* strains were grown aerobically at 37°C with shaking in LB medium (solid or liquid as needed) supplemented with antibiotics (100 μg/ml ampicillin and/or 50 μg/ml chloramphenicol) as necessary. Non-coliform bacteria were grown aerobically at 32°C with shaking in tryptic soy medium (solid or liquid as needed). Bacterial strains were stored as glycerol stocks at -80°C and streaked out on solid media as needed. All phages were stored at 4°C until needed.

Cloning

Expression plasmids pSOC6H and pClickSOC6H were constructed from the pET 11a backbone (Invitrogen, Burlington, MA, USA) using HiFi DNA assembly (NEB, Ipswitch, MA, USA). RB69 SOC was amplified from genomic DNA and modified to include a C-terminal 6X-His tag and an N-terminal amber codon using Q5 PCR master mix (NEB) and the primers in Table S2 (designed with the NEBuilder tool). Plasmids were amplified in E. cloni® 10G Electrocompetent Cells

(Lucigen, Middleton, WI, USA), purified with the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA), and validated by Sanger DNA sequencing.

Wild-type SOC expression and purification

pSOC6H was transformed into BL21(DE3) *E. coli* for expression. Cultures were grown to an OD_{600} of 0.5 in 2xYT medium supplemented with 10 μg/ml ampicillin and induced with 400 μM isopropyl-β-D-1 thiogalactopyranoside (IPTG, Thermo Fisher Scientific, Waltham, MA, USA) for 15-16 hours at 30°C. Cells were pelleted by centrifugation (3,260 × *g*, 15 minutes) flash frozen in an ethanol/dry ice bath, and lysed by incubating with 5 ml B-PER complete (Thermo Fisher, Waltham, MA, USA) per gram of wet cell pellet for 15 minutes. Lysates were diluted 10-fold in wash buffer (500 mM NaCl, 25 mM imidazole [pH 8.0], 50 mM sodium phosphate [pH 8.0]). After clarifying the lysate by centrifugation (3,260 × *g*, 1 hour), the supernatant was batch-bound for 1 hour to 500 μl HisPur Cobalt Resin (Thermo Fisher) equilibrated in 1 ml wash buffer. Resin was collected by centrifugation (700 × *g*, 2 mintues) and washed six times in excess wash buffer, with resin pelleted after each wash. Protein was eluted in 2 ml elution buffer (wash buffer with 150 mM imidazole) and dialyzed against excess dialysis buffer (150 mM NaCl, 50 mM sodium phosphate [pH 8.0]) for 3 hours, after which the buffer was changed and dialysis continued for 18 hours more. The resulting purified protein was analyzed by SDS-PAGE, quantified using Bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA, USA), and stored at 4°C until further use.

Click SOC expression

pClickSOC6H and pEvol pPrpRS were co-transformed into BL21(DE3) *E. coli* for expression. Cultures were grown from single colonies in ampicillin and chloramphenicol-supplemented minimal glycerol medium as previously described[50] in a fed-batch fermentor (BioFlo 2000, New Brunswick Scientific, Edison, NJ, USA). When initial carbon was exhausted (indicated by reduced agitation speed), glycerol feeding proceeded exponentially for 18 hours with a fixed growth rate of 0.1 h^{-1} calculated as previously described[51]. After 14 hours feeding, 1 mM of the artificial amino acid prp (Chem-Impex International, Wood Dale, IL, USA) was added to the culture. Expression of prp tRNA synthetase was induced by addition of 0.02% L-+-arabinose after 16 hours of feeding. Expression of Click SOC was induced by addition of 1 mM IPTG and 0.2% yeast extract after 18 hours of feeding. After IPTG induction, expression continued for 21 hours at 30°C with pH-stat feeding as previously described[51]. Cells were pelleted by centrifugation (4,000 × *g,* 30 minutes), flash frozen in an ethanol/dry ice bath, and stored at -80°C until further use. For purification, pellets were thawed overnight at 4°C and mixed with 3 ml lysis buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, 25 mM imidazole [pH 8.0], 25% sucrose, 0.5 mg/mL lysozyme, 40 µg/mL DNAse I, 0.5% IGEPAL) for 30 minutes at room temperature. Lysates were clarified by centrifugation (3,260 × *g*, 1 hour) and purification proceeded as described above.

Fluorescent cycloaddition assays

Approximately 90 ng SOC was mixed to a final volume of 200 µL with the following: 37.5 mM NaCl, 12.5 mM sodium phosphate [pH 8.0], 6 M guanidinium, 250 µM CuSO4, 200 µM 3-azido-7-hydroxycoumarin (MarkerGene, Eugene, OR, USA), 250 µM Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine, and 1 mg copper wire as a reducing agent. SOC was replaced with 50 nM prp for a positive control. The cycloaddition reaction proceeded at 37°C for 4 hours. Fluorescence (ex: 404 nm / em: 477 nm) was measured with a spectrophotometer (BioTek, Winooski, VT, USA).

Phage propagation

Phage particles were added to logarithmically growing ($OD_{600} \approx 0.5$) DH5α *E. coli* with a 0.1 multiplicity of infection (MOI). Infection proceeded at 37°C with shaking (150 rpm) for 4 hours, at which point there was visible clearance of the culture. Cell debris was pelleted by centrifugation (3,260 × *g*, 15 minutes) and the resulting clarified lysates were incubated at room temperature for 16-18 hours. Phages were pelleted by centrifugation (30,000 × *g*, 3 hours) resuspended in 15 ml sterile phosphate-buffered saline (PBS), and enumerated using standard plaque-count techniques.

Directed self-assembly of SOC on phages and western blots

Approximately 200 ng of SOC was mixed with 10 log pfu of phages in 1 ml assembly buffer (75 mM NaCl, 50 mM sodium phosphate [pH 7.0], 1 mM $MgSO_4$) and incubated for 90 minutes at 4°C. Phages were pelleted by centrifugation at 16,000 × *g* for 40 minutes. Pellets were washed twice in excess buffer, centrifuging in the same conditions after each wash, and then resuspended to a final volume of 16 µl. Reducing SDS-PAGE sample buffer (BioRad) was added to a final concentration of 1X and samples were boiled for 5 minutes before being run on 4-20% gradient SDS-PAGE gels. Protein was transferred to nitrocellulose membranes with a semi-dry process and blocked in 5% nonfat dry milk powder. SOC was detected by alkaline phosphatase-conjugated antibody against the 6X His tag (Invitrogen).

Synthesis of phage-based bioaffinity nanobots

Azide-coated magnetic nanoparticles (MNPs) (TurboBeads, Zurich, SUI) were stored in PBS and protected from light. To make 1 ml click-immobilized phages, 9 log pfu of NRGp17 was mixed with 10^{10} MNPs, SOC (20 ng Click-SOC or 2 µg WT SOC), 1 mM $CuSO_4$, and 1 mg copper wire in 1 ml assembly buffer supplemented with 0.1% Tween-20. Reactions were incubated 16-18 hours

at 4°C, after which beads were collected by incubation against a magnet for 15 minutes. While still on the magnet, beads were washed three times in excess buffer and resuspended to a final volume of 1 ml in fresh buffer. The resulting nanobots were stored at 4°C.

Small-scale detection of *E. coli*

Single colonies of *E. coli* (ECOR#13) were grown for 16-18 hours in 10 ml total culture volume. Serial dilutions were performed in sterile PBS and cultures were enumerated using standard plate counting techniques. 100 µl aliquots of 10^{-6} (3-4 log cfu/ml) and 10^{-8} (1-2 log cfu/ml) dilutions were placed in separate tubes and spiked with 2 µl phage-decorated beads. 100 µl sterile PBS was used as a 0 cfu control. Other controls included 2 µl of free NRGp17[38] at 5 log pfu/ml, 2 µl of unconjugated MNPs at 10^5 ml^{-1}, and 2 µl of phage-decorated MNPs prepared as above but with Click SOC replaced with additional assembly buffer. Samples were statically incubated for 15 minutes to allow phages to bind to bacteria, then moved to a magnetic rack and incubated for 15 minutes more to collect bacteria. Supernatant was aspirated and beads resuspended in 100 µl LB medium containing 7 log pfu/ml NRGp17 to ensure lysis of all captured cells. Resuspended samples were incubated with shaking for 6 hours at 37°C and then vacuumed through single wells of a 384 well filter plate. 50 µl freshly prepared NanoGlo luciferase assay substrate (Promega, Madison, WI, USA) was added to each well and luminescence was read on the BioTek spectrophotometer.

Large-scale detection of *E. coli*

Starter culture and dilutions were prepared as described above. 100 ml samples of autoclaved tap water were prepared in disposable coliform test bottles (Corning Inc.) and spiked with either 20 µl of the 10^{-6} *E. coli* (ECOR#13) dilution (1.3-2.3 log cfu) or 100 µl of the 10^{-8} *E. coli* dilution (0-1

log cfu), or else left sterile. 5 ml 20X LB broth was added to each sample and bottles were incubated with shaking at 37°C for 3 hours. 2 ml phage-decorated beads, prepared as described above, were added to each bottle followed by a 10-minute incubation. Samples were poured into square petri dishes to maximize surface area and incubated against a custom-built magnetic rig (bar magnets adhered to 10 cm square supports) for 10 minutes. Supernatants were aspirated and discarded, dishes removed from magnets, and beads were resuspended in 500 µl LB broth containing 10^7 pfu/ml NRGp17. Resuspended beads were incubated with shaking for 3 hours at 37°C followed by filtration and detection as described above.

Competitive detection of *E. coli*

Single colonies of non-coliform, water-associated bacteria (*Listeria monocytogenes, Pseudomonas aeruginosa, Sphingopyxis alaskiensis*) were grown in 10 ml liquid culture for 24 hours. Serial dilutions were performed in sterile PBS and cultures were enumerated using standard plate count techniques. *E. coli* (ECOR#13) dilutions were prepared as described above. Cocktail cultures were prepared by combining ~3 log cfu/ml of each non-coliform strain. Detection samples included non-coliform cocktail spiked with *E. coli* , *E. coli* with no competitors, cocktail with no added *E. coli* , and sterile PBS. 100 µl samples were processed as described above for small-scale detection samples.

Magnetic nanoparticle size determination

Magnetic nanoparticles in aqueous suspension at a concentration of ~10^9 ml^{-1} were analyzed using a Zetasizer (Malvern Pananalytical, Malvern, UK). Dynamic light scattering data are shown in Table S9.

Statistics and data processing

All quantitative assays were performed with 3-4 technical replicates and at least 1 biological replicate. Not all biological replicates included technical replicates; these were not included in the statistical analyses and are reported with the raw data in Tables S3-S8. Pairwise comparisons were done using two-tailed, unpaired T-tests. Small-scale *E. coli* (ECOR#13) tests, including competitor assays, were analyzed in JMP Pro 14.0.0 (Cary, NC, USA) using a standard least-squares model. Large-scale *E. coli* (ECOR#13) detection and fluorescent cycloaddition were analyzed in GraphPad Prism (La Jolla, CA, USA) using standard one-way ANOVA. Significance was determined with a cutoff of $p < 0.05$.

SUPPORTING INFORMATION

Table S1 lists all phage and bacteria strains used in this study and their sources. Table S2 lists all primers used in this study, their sequences, and their purposes. Table S3 provides the raw data displayed in Figure 3a. Table S4 provides the raw data displayed in Figure 4. Table S5 provides the raw data displayed in Figure 5a. Table S6 provides the raw data displayed in Figure 5b. Table S7 provides the raw data displayed in Figure 6. Table S8 provides additional biological replicates of large-scale detection. Figure S1 gives an overview of the genetic system used to construct Click SOC. Figure S2 shows SOC purification data.

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H.S.Z., J.M.G, and S.R.N. conceived the study, designed all experiments, and wrote the manuscript. M.M.D. constructed NRGp17. H.S.Z. performed all other experiments and analyzed the data. J.M.G. and S.R.N. supervised the study. All authors reviewed the manuscript before submission.

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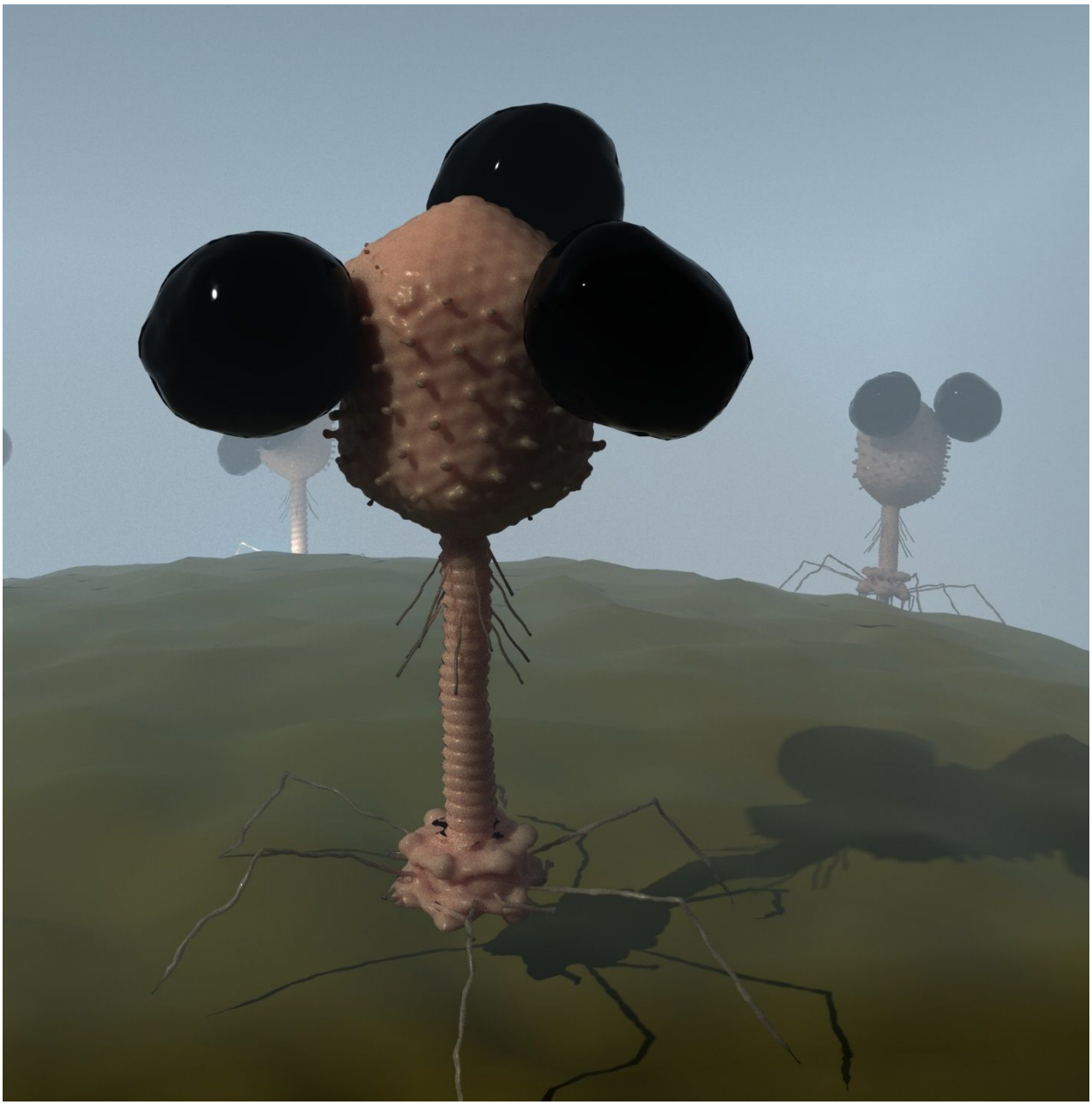
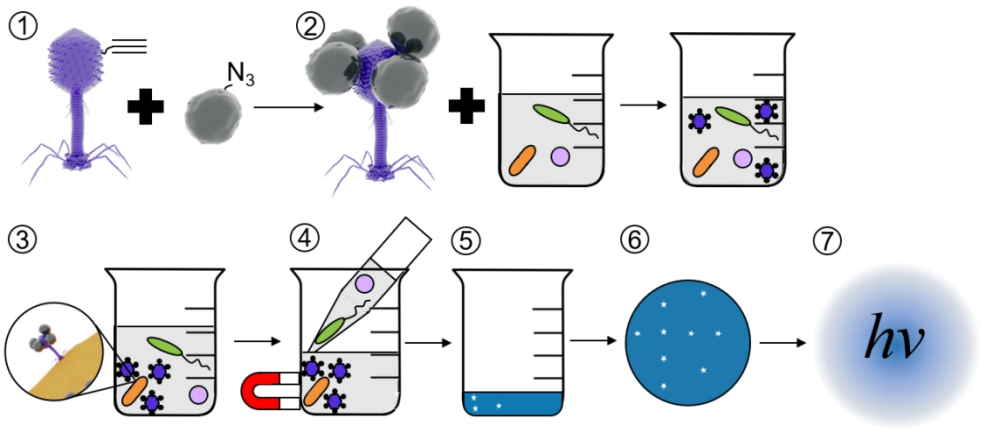


TOC



Schematic of *E. coli* detection assay. Self-assembled alkynylated T4 Nluc CBM phages are conjugated to azide-coated magnetic nanoparticles (MNPs) (1) using click chemistry. The resulting magnetic phage-based bioaffinity nanobot is added to samples of environmental water (2), where the phages bind specifically to *E. coli* (orange rods, 3). Beads, along with phages and E. coli are collected with a magnet and excess volume is aspirated (4). Infection cycle completes (5), releasing Nluc-CBM into solution. Samples are filtered (6) on cellulose to immobilize Nluc-CBM and luminescence is measured (7) to detect *E. coli*. Please note that the schematics in this figure are not to scale. T4 Nluc CBM is approximately 200 nm long and 900 nm in diameter. MNPs are approximately 15 nm in diameter(Table S9).

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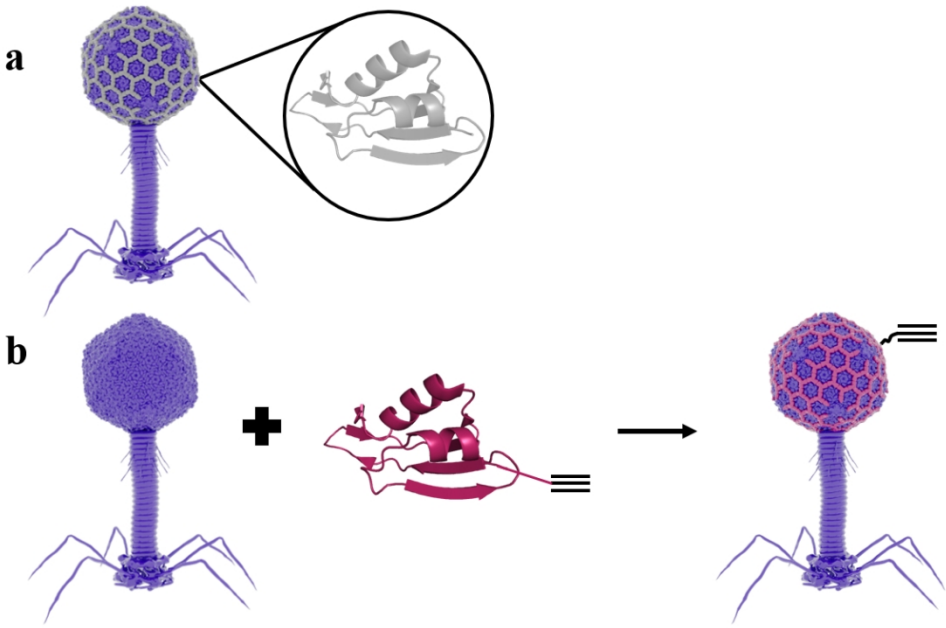
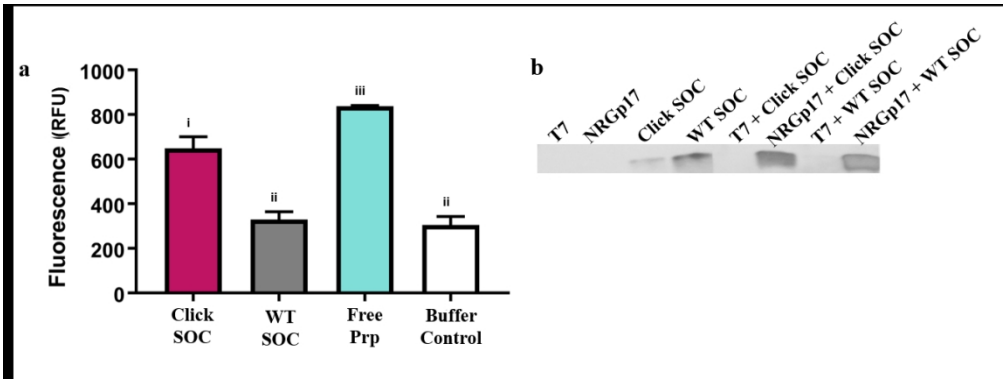


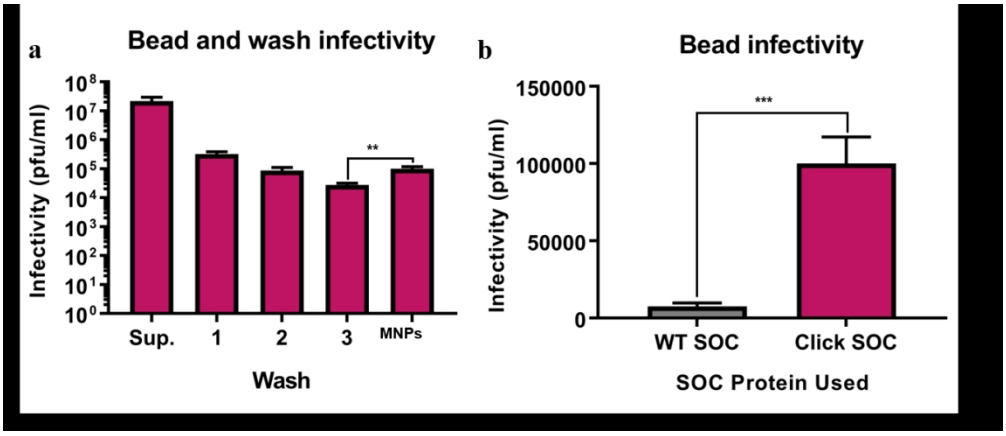
T4 phage capsid alkyne decoration strategy and Click SOC construct design. a) Schematic of WT SOC (grey, PDB ID 3IGE) assembly on the T4 capsid (PDB ID 5VF3). b) A SOC with a alkyne-containing unnatural amino acid is incorporated into the phage capsid to allow for click chemistry conjugation. Each phage capsid can accommodate 870 SOC proteins. Click-SOC self-assembled on T4 Nluc CBM (Δ*soc*). Click-SOC is shown in red with an alkyne schematic.

202x135mm (150 x 150 DPI)



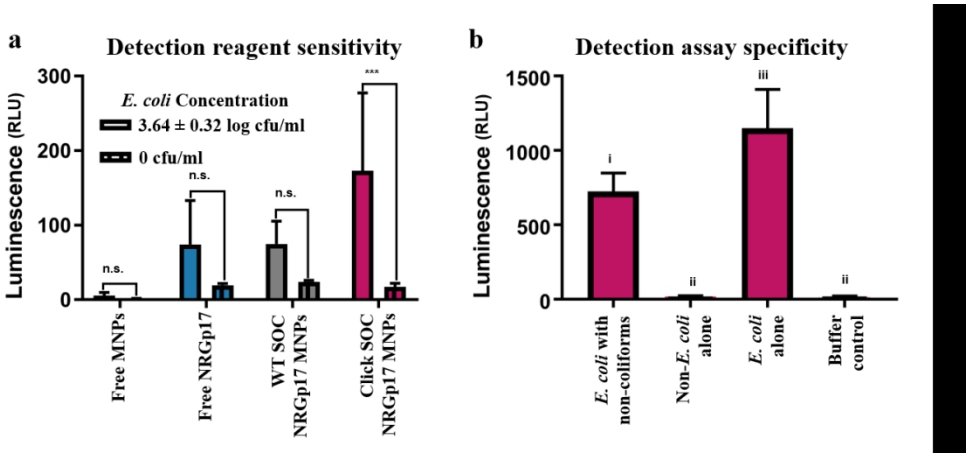
Click SOC has alkyne functionality and retains self-assembly properties. a) Fluorescence of 3-azido-7-hydroxycoumarin after click reaction. Click SOC has significantly higher fluorescence than WT SOC or the buffer control, indicating the successful introduction of available alkyne groups. Error bars indicate standard deviation of four technical replicates. Letters indicate significance by one-way ANOVA and Tukey's multiple comparison test ($p < 0.05$). b) Western blot against the C-terminal 6X His tag on WT SOC and Click SOC before and after self-assembly on phages confirms Click SOC has the same T4 self-assembly properties as WT SOC. T7 was used as a negative control for self-assembly. The raw data used to construct this figure are reported in Table S3.

227x85mm (150 x 150 DPI)



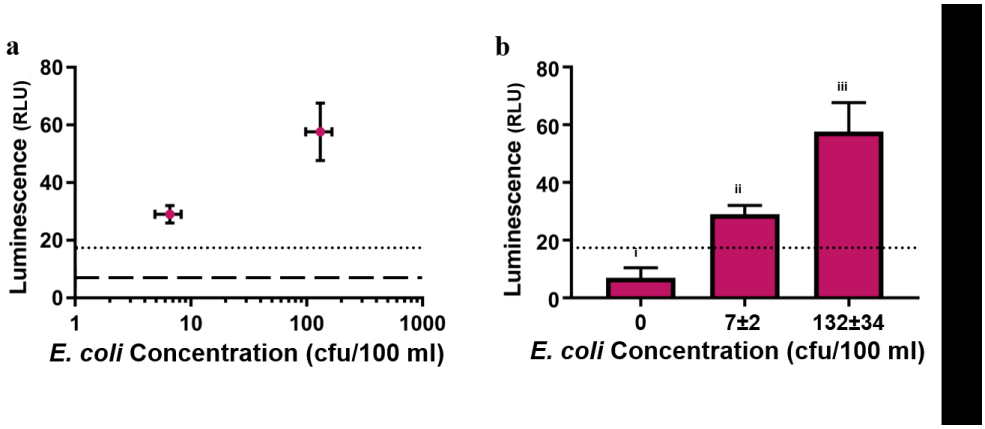
Immobilized phages retain infective properties after magnetic capture. a) The resuspended nanobots are significantly more infective than the final wash, indicating that phages have been magnetically captured and resuspended during the washing process. b) Infectivity of MNPs conjugated to NRGP17 by either WT SOC or Click SOC. Click SOC conjugation results in significantly higher infectivity, indicating a stronger conjugation between the phage and the bead. Error bars indicate standard deviation of three technical replicates. Asterisks indicate significance (** = $p < 0.01$, *** = $p < 0.001$) by unpaired two-tailed t-test. The raw data used to construct this figure are reported in Table S4.

230x98mm (150 x 150 DPI)



MNPs with click-immobilized NRGp17 are a specific, sensitive detection tool. a) Small-scale *E. coli* (ECOR#13) detection with no pre-enrichment. 100 µl samples were treated with 5 log ml-1 unconjugated azide-coated MNPs, 5 log pfu/ml free NRGp17, NRGp17 conjugated to MNPs via WT SOC, or Click NRGp17 conjugated to MNPs. Only the latter gives a signal significantly higher than the baseline luminescence. b) Small scale detection of 4.01 ± 0.15 log cfu/ml *E. coli* with no pre-enrichment in pure and mixed culture. Non-coliform bacteria included 3.27 ± 0.33 log cfu/ml *Pseudomonas aeruginosa*, 3.82 ± 0.22 log cfu/ml *Listeria monocytogenes*, and 3.23 ± 0.07 log cfu/ml *Sphingopyxis alaskiensis*. Error bars indicate standard deviation of four technical replicates. Bacterial concentration reported as mean ± standard deviation of 12 plate counts. Letters and stars indicate significance ($p < 0.05$; n.s. = $p > 0.05$; *** = $p < 0.001$) by one-way ANOVA and Tukey's multiple comparison test. Raw data used to construct this figure are reported in Tables S5 and S6.

230x103mm (150 x 150 DPI)



Phage-based bioaffinity nanobots can detect <10 cfu *E. coli* in 100 ml tap water within 7 hours. Luminescence error bars indicate standard deviation of 3 technical replicates. Dotted lines indicate LOD (3 standard deviations above mean baseline luminescence). a) *E. coli* (ECOR#13) concentration plotted against luminescence. Dashed line indicates baseline luminescence. Concentration error bars indicate standard deviation of 12 plate counts. b) Luminescence values obtained from different *E. coli* concentrations. Letters indicate significance ($p < 0.05$) by one-way ANOVA and Tukey's multiple comparison test. Bacterial concentration reported as mean ± standard deviation of 12 plate counts. Raw data used to construct this figure are reported in Table S7.

226x95mm (150 x 150 DPI)