1 **Immobilization of antimicrobial core-shell nanospheres onto silicone for**

2 **prevention of *Escherichia coli* biofilm formation**

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15

1 **Abstract:**

2 *Escherichia coli* (*E. coli*) strains are among the most frequently isolated

3 microorganisms in urinary tract infections able to colonize the surface of urinary

4 catheters and form biofilms. These biofilms are highly resistant to antibiotics and host

5 immune system, resulting in increased morbidity and mortality rates. Strategies to

6 prevent biofilm development, especially via restricting the initial stages of bacteria

7 attachment are therefore urgently needed. Herein, a common urinary catheter material -

8 polydimethylsiloxane (PDMS) - was covalently functionalized with antibacterial

9 aminocellulose nanospheres (ACNSs) using the epoxy/amine grafting chemistry. The

10 PDMS surface was pre-activated with (3-glycidyloxypropyl)-triethoxysilane to

11 introduce epoxy functionalities prior to immobilization of the intact ACNSs via its

12 amino groups. The AC biopolymer was first sonochemically processed into NSs

13 improving by up to 80 % its potential to prevent the *E. coli* biofilm formation on a

14 polystyrene surface. The silicone surface decorated with these NSs demonstrated

15 efficient inhibition of *E. coli* biofilms, reducing the total biomass when compared with

16 pristine silicone material. Therefore, the functionalization of silicone-based materials

17 with ACNSs shows promise as potential platform for prevention of biofilm-associated

18 infections caused by *E. coli*.

19

20 **Key words:** *Escherichia coli*, core-shell nanospheres, sonochemistry, silicone

21 functionalization; epoxy-amine grafting, antibiofilm strategies

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1 Introduction

Bacterial biofilms are formed when unicellular organisms come together to develop a well-organized community of cells attached to a solid surface and encased in an extracellular polymeric matrix. When growing in the biofilm phenotype, bacteria are able to survive in hostile environments, acquire increased antibiotic tolerance and resist the clearance by the host immune system [1–3]. Indwelling medical devices are prone to bacterial colonization, causing difficult-to-treat biofilm-induced infections that usually result in long-term hospitalizations, increased health care costs, need for surgical removal of the contaminated device and affected areas, and even death [4,5].

A number of factors render catheters susceptible to microbial contaminations. First, catheter insertion often compromises the skin protective barrier, providing a direct route for opportunistic bacteria to bypass the first line of human immunity defence and establish infection. Moreover, upon insertion, the outer surface of the catheter is quickly covered by host proteins, e.g. fibronectin, fibrinogen, collagen, lipids, polysaccharides and inorganic salts, forming the so called "surface conditioning layer". This layer modifies the surface properties and facilitates the attachment [6] of microorganisms present in the ambient surrounding the site of insertion (flora) [7]. Finally, patients who possess the greatest need for implanted medical devices are often immunocompromised and therefore are more susceptible to bacterial infections [8].

E. coli is one of the predominant inhabitants of the human gastrointestinal tract and perineum that most often causes urinary tract infections (UTIs). Specific pathogenic *E. coli* strains may express a variety of adhesins and easily colonize the bladder or other parts of the urinary tract as well as the surface of urinary catheters causing difficult to treat infections [9]. After the initial colonization the natural progressive step in bacteria

1   lifestyle is the establishment of biofilm. The biofilm then serves as a growing, often

2   antibiotic-resistant reservoir that seeds infection throughout the host. In line with this,

3   there is an urgent need for novel efficient prophylaxis strategies to prevent biofilm-

4   associated drug-resistant bacterial infections.

5   To this end, we functionalized polydimethylsiloxane (PDMS) the common material for

6   manufacturing of urinary catheters with antimicrobial aminocellulose nanospheres

7   (ACNSs). The strategy is based on our previous findings that ACNSs, generated using a

8   one-step sonochemical process, inhibit the *E. coli* growth by a mechanism that includes

9   the disruption of bacterial membrane, assigned to the high cationic charge after the

10 nanospherization process [10]. In addition, ACNSs prevent the formation of bacterial

11 biofilms on urinary catheters when deposited by layer-by-layer technology [11]. The

12 herein covalent functionalization approach involves treatment of the PDMS surface

13 with epoxy functional groups followed by the immobilization of ACNSs using the well-

14 known epoxy-amine chemistry. The curing reactions of epoxy resins with aliphatic

15 amines have been widely used for the development of coatings due to the fact that these

16 compounds react easily at room temperature [10,12–15]. The ultimate goal of the study

17 was to provide the proof of concept for permanent functionalization of silicone-based

18 indwelling medical devices as an efficient strategy to counteract the *E. coli* attachment

19 and avoid biofilm formation.

20

1   **Materials and methods**

2   PDMS silicone sheets, designated according to ASTMD 1418 were kindly provided by

3   Degania Silcone Ltd. (Israel). Proficient in biofilm formation *E. coli* ATCC 25922

4   bacterium was obtained from American Type Culture Collection ATCC (LGC

5   Standards S.L.U, Spain). Live/Dead$^{®}$ BacLight™ bacterial viability kit (Molecular

6   probes L7012) was purchased from Invitrogen, Life Technologies Corporation (Spain).

7   Microcrystalline cellulose (Fluka, Avicel PH-101), dried at 105 °C for 2 h, was used for

8   the preparation of the 6-deoxy-6-(ω-aminoethyl) aminocellulose derivative. All other

9   chemicals were purchased from Sigma-Aldrich (Spain) and used without further

10   purification.

11

12   **Cellulose Amination**

13   The cationic derivative of cellulose, 6-deoxy-6-(ω-aminoethyl) aminocellulose (AC,

14   ~15kDa), was synthesized from microcrystalline cellulose via a tosyl cellulose

15   intermediate, as previously described [16]. Briefly, cellulose was dissolved in N,N-

16   dimethylacetamide/LiCl (DMA/LiCl) and allowed to react with p-tosyl chloride (TosCl)

17   in the presence of triethylamine (Et3N) at 8-10 °C. The degree of substitution of the

18   tosylate groups (DSTos) was adjusted by the application of different molar ratios of p-

19   tosyl chloride and triethylamine. A total of 20 equivalents of ethylenediamine were

20   added, and the temperature of the reaction mixture was increased to 100 °C and stirred

21   for 3 h. The product was isolated by precipitation in 200 mL of water. The precipitate

22   was filtered off and washed four times with 150 mL of isopropanol and four times with

23   150 mL water. The product was dried in vacuum at 40 °C.

24

1 **Preparation of aminocellulose nanospheres**

2 ACNSs were prepared as previously described [10]. Briefly, a two-phase solution

3 containing 70 % of 1 g/L AC aqueous solution, pH 6, and 30 % of commercial

4 sunflower oil (organic phase) was prepared and placed into a thermostated (8 °C ± 1 °C)

5 sonicator cell. The ACNSs were generated using a high-intensity Vibra-Cell VCX 750

6 ultrasonic processor (Sonics and Materials, Inc., U.S.A.) with 20 kHz Ti horn at 35 %

7 amplitude. The bottom of the probe was positioned at the aqueous-organic interface,

8 employing an acoustic power of ~0.5 W/cm^3 for 3 min using an ice-cooling bath to

9 maintain the reaction at low temperature (8-11 °C). The resulted suspension was kept at

10 4 °C for 24 h and the non-reacted organic solvent was removed by three washing cycles

11 with water and centrifugation at 800 rpm for 15 min.

12

13 **Incorporation of ACNSs onto silicone material**

14 PDMS stripes (9 x 3.2 cm^2) were washed with 0.5 % (w/v) sodium dodecyl sulfate

15 (SDS), water and ethanol. Then, the strips were functionalized with 5 % (v/v) 3-

16 (glycidoxypropyl) trimethoxysilane (GOPTS) solution in 96 % EtOH for 12 h at room

17 temperature. The samples were washed with ethanol, dried at 85 °C for 2 h and stored in

18 the dark for at least 2 days prior to functionalization with ACNSs. The presence of

19 epoxy groups on the surface was confirmed by development of characteristic pink

20 colour in a 4-(para-nitrobenzyl) pyridine (p-NBP) test. Briefly, treated and non-treated

21 silicone samples were incubated in 5 mL of 200 mM p-NBP solution in methoxyethanol

22 for 30 min at 80 ºC.

23 For epoxy/amine curing reaction, the pre-activated silicone strips were cut and placed in

24 15 mL test tubes containing 5 mL of 0.1 mg/mL ACNSs solution previously

1 conditioned at pH 3 and 6. Then, the tubes were transferred in a water bath at 60 ºC and

2 kept overnight with shaking. Afterwards the silicone stripes were washed three times

3 with distilled H_2O, until no spheres were released into the washing solution, verified

4 optical microscopy and dynamic light scattering (DLC) and stored at 4 °C for further

5 analysis.

6

7 **Surface characterization**

8 The deposition of ACNSs on the silicone material was assessed by ATR-FTIR using a

9 Spectrum 100 FT-IR spectrometer (Perkin Elmer, USA). The ATR-FTIR spectra of

10 pristine, epoxy treated and ACNSs coated silicone materials were collected in the range

11 over 4000 to 625 cm^{-1}. All the spectra were obtained after 64 scans at 4 cm^{-1} resolution

12 and the data was analyzed using essential eFTIR - 3.00.019 software. The shape and

13 morphology of immobilized NSs was further assessed with a scanning electron

14 microscope (SEM) using a cross-beam workstation (Zeiss Neon 40) with Focused Ion

15 Beam FIB/SEM beams for sample observation.

16

17 **Inhibition of *E. coli* biofilms**

18 Prior to immobilization of the spheres on silicone samples, the biofilm inhibition

19 activity of the ACNSs was evaluated and compared to non-processed AC in solution.

20 For optimal biofilm growth, fresh single *E. coli* colony was inoculated in 5 mL tryptic

21 soy broth (TSB) and cultured overnight at 37 °C with shaking (230 rpm). 100 µL of *E.*

22 *coli* cells were mixed with 100 µL ACNSs or ACsol at 0.5 mg/mL in a 96-well plate

23 (polystyrene, surface-treated) and then cultured for 24 h at 37 °C. After incubation, the

24 mixtures were withdrawn from the wells and the surfaces further washed with sterile

1 distilled H_2O, fixed by heat (60 °C for 60 min) and the total biofilm mass was measured

2 with 0.01 % (w/v) crystal violet solution as previously described [17].

3 *E. coli* biofilm mass on ACNSs treated silicone surfaces was also assessed. The

4 bacterial culture was diluted to OD_{600} = 0.01 in TSB and 1 mL of the inoculum was

5 incubated with the silicone samples (1 x 1 cm^2) in a cell-culture 24-well plate. The

6 biofilms were allowed to grow statically for 24 h at 37 ºC and the total biomass was

7 assessed using crystal violet as above described.

8

9 *E. coli* **cells viability in biofilms**

10 *E. coli* biofilms were grown on silicone samples and polystyrene surface as described

11 above and then analyzed with Live/Dead® BacLight™ bacterial viability kit. Following

12 24 h of incubation, the biofilms were washed with sterile sodium chloride (NaCl, 0.9 %)

13 pH 6.5 and stained for 15 min using a mixture of two fluorescent dyes - Syto 9® and

14 propidium iodide (1:1). The *E. coli* biofilms were then observed using fluorescence

15 microscopy at $\lambda_{exc}/\lambda_{em}$= 480/500 nm for Syto 9 labeling in green the nucleic acid of all

16 bacteria with intact and damaged membranes, and at $\lambda_{exc}/\lambda_{em}$= 490/635 nm for

17 propidium iodide quenching the green fluorescence of Syto 9 dye after penetration into

18 the damaged cells and staining the dead bacteria in red.

19

20

1 **Results and discussion**

2 Due to their intrinsic biocompatibility and durability, the PDMS materials,

3 commercially known as silicones, are widely used for manufacturing medical devices

4 such as urinary catheters, surgical incision drains and respiratory devices. In terms of

5 infection prevention, silicone-based urinary catheters have been reported to possess

6 superior to latex and polyvinyl chloride (PVC) performance and although being more

7 expensive these are the materials of choice for indwelling catheterization

8 [18]. Nevertheless, the presence of microorganisms in the surrounding environment of

9 catheters ultimately leads to their contamination. A variety of bacterial species are able

10 to colonize silicone catheters and develop drug resistant biofilms, among which, Gram-

11 negative *E. coli* is one of the most common species causing UTIs [19].

12 In order to obtain a silicone-based material with antibiofilm properties against *E. coli*

13 we developed a surface functionalized with ACNSs, previously shown to eradicate

14 planktonic *E. coli* cells [15]. The ACNSs were generated using a sonochemical method

15 first reported by Suslick and Grinstaff [20] and recently used to synthetize biopolymer-

16 based nano/microcapsules [10,21]. This method allowed for the preparation of oil filled

17 spheres from an aqueous solutions of aminocellulose, a highly cationic biopolymer

18 obtained from microcrystalline cellulose via a tosyl cellulose intermediate reaction [16]

19 (Scheme 1). The sonochemical-induced nanospheres formation is reported to be assisted

20 by effective intermolecular interactions (hydrogen bonding, van der Waals,

21 hydrophobic, and electrostatic interactions) that occur during the emulsification process

22 in which the aminocellulose molecules, initially present in the aqueous phase, localize

23 at the interface of the droplet, thus, generating a sphere with a biopolymeric shell and an

24 oil core [20].

1   **Scheme 1**

2   As previously reported [10], the use of this technique for the formation of ACNSs

3   resulted in a stable dispersion with spheres of an average hydrodynamic diameter of 268

4    $\pm$ 7 nm, narrow size distribution and extremely high ζ-potential, $+103 \pm 2$ mV. Besides

5   an extremely high stability, such high cationic charge of the spheres was also found to

6   be the main reason for the efficient interaction with the negatively charged cell

7   membrane and consequent bacterial killing. Apart from the killing effect this strategy

8   presents other advantages in terms of overcoming the development of microbial drug

9   resistance due to the fact that it targets the membrane of bacteria, which is highly

10   evolutionarily conserved and therefore unlikely to be changed by a single gene

11   mutation. In fact, different macromolecules processed in the form of nanospheres and

12   nanoparticles were previously suggested as superior antibacterial agents against bacteria

13   in both planktonic form and within biofilms mainly because their small size, charge

14   concentration and high surface reactivity [22]. For that reason we have also tested the

15   ACNSs for their capacity to prevent biofilm formation and compared it with AC in

16   solution (ACsol). The *E. coli* biofilm was grown on a polystyrene surface together with

17   ACNSs or ACsol and further analysed for biofilm inhibition. Characteristic biofilm

18   structure of *E. coli* cells was observed when the biofilm grew in contact with ACsol,

19   whereas few bacterial clusters were formed in the presence of bactericidal spheres

20   (Figure 1 B). The total biofilm mass decreased by up to 80 % in the presence of NSs,

21   while almost no significant reduction was observed in the case of 0.5 mg/mL non-

22   processed ACsol (Figure 1 A). The spherical shape and large surface area of the ACNSs

23   is believed to be the main reason for the prevention of bacterial biofilms by penetrating

24   more easily than the ACsol and by providing better contact with the matrix encased

1 microorganisms, thus allowing the eradication of biofilms and even inducing cells death

2 [10].

3 **Figure 1**.

4 This study further exploited the possibility for coating PDMS with the produced

5 polycationic NSs in order to engineer novel antibiofilm surfaces. A previous study

6 performed in our group has proven the ability of these ACNSs, incorporated onto

7 PDMS using layer-by-layer technique, to impart antibiofilm and antibacterial properties

8 to the surface of silicone [11]. To obtain the same effect, PDMS surface was initially

9 functionalized with GOPTS in order to obtain a surface with pendant reactive epoxy

10 groups for interaction with the amino bearing ACNSs.

11 Despite the fact that PDMS has been reported as an inert material for functionalization,

12 possessing scarcely distributed reactive hydroxyl groups on the surface, some authors

13 have described a chemisorption phenomenon that allows for the formation of an

14 oligomeric siloxane layer on the silicone surface [13]. In this process both PDMS and

15 GOPTS are hydrolysed in the presence of water, resulting in the generation of hydroxyl

16 groups that further may interact via hydrogen bonding, thus leading to the

17 functionalization of the PDMS with epoxy pendant groups (Scheme 2). Since the epoxy

18 groups could not be identified by FTIR (results not shown), the success of the used

19 functionalization strategy was checked with p-NBP, a dye commonly used to

20 spectrophotometrically identify the presence of epoxy groups [23]. The generation of a

21 pink coloured complex after the reaction with the p-NBP confirmed the presence of

22 epoxy groups (Scheme 2) [24].

23 **Scheme 2**

1   The addition of epoxy functionalities provided a reactive site for further immobilization

2   of amino-containing compounds. This reaction occurs via a nucleophilic attack of the

3   amine nitrogen on the terminal carbon of the epoxy function. The mechanism is a S_N^2-

4   type II [15], in which a primary amine can react twice with two epoxy group while a

5   secondary amine can react only once [12]. The amino groups from our AC biopolymer

6   and within ACNSs are primary amines and thus able to react with the epoxy group on

7   the functionalized surface through the active amine hydrogen (Scheme 2).

8   The pre-functionalization of silicone with epoxy groups was essential for the

9   immobilization of ACNSs. The infrared spectra of the epoxy-functionalized silicone

10 treated with ACNSs, showed the characteristic for amine groups and the N-H stretching

11 vibrations peaks at around 1650 cm^{-1} and 2900 cm^{-1}, suggesting that ACNSs were

12 indeed present at the surface of silicone (Figure 2). Without the epoxy pre-treatment, the

13 infrared spectra of the pristine and treated with ACNSs silicones are the same,

14 indicating the absence of the biopolymer on the surface. The silicon pre-functionalized

15 with epoxy groups also shown a spectra without

16 **Figure 2**

17 Therefore, the epoxy-treated silicone was further functionalized with antimicrobial

18 ACNSs previously dispersed in a solution at pH 3 and pH 6. These two pHs were used

19 to manipulate the properties of the amino groups of the spheres, namely: at pH 3 the

20 amino groups are mainly protonated ($-NH_3^+$) and thus more reactive towards negatively

21 charged surfaces, while at pH 6 the amino groups are more nucleophilic, which should

22 favour the epoxy-amino curing reaction. SEM images have shown that the

23 immobilisation of ACNSs was successful when both pHs were used, importantly

24 revealing the presence of intact spheres on the surface. The size of the spheres

corresponds to the size measured in the dispersion. The bigger spheres that appear in the SEM images are air-filled microbubbles, shelled by oil-filled ACNSs (smaller spheres in the images). This pattern has been described to occur in sonochemically generated dispersions from polysaccharide conjugates (Figure 3) [25]. The immobilization of intact nanostructures, e.g. core/shell NSs with a soft core, is advantageous, since the added value of processing polymers into nanostructures is transferred to the functionalized materials/surfaces [11]. Important for our study observation is that considerably more NSs were fixed on the epoxy-treated silicone when the immobilization reaction was performed at pH 6 because of the improved nucleophilicity of the amine groups at these conditions. For this reason, only the silicone treated with the spheres at pH 6 was used for further experiments.

Figure 3

The functionalized silicone surface was then tested for preventing *E. coli* biofilm formation. *E. coli* is one of the most frequently isolated microorganisms in catheter-associated UTIs [9]. It is a highly versatile bacterium ranging from harmless intestinal inhabitant to deadly pathogen, including common colonizers of medical devices, small intestine and the urethra [26]. On the surface of urinary catheters *E. coli* may establish biofilms and cause difficult to treat urogenital infections.

Figure 4

Since ACNSs were found to be efficient in preventing the *E. coli* biofilm growth on polystyrene surface, we assessed the ACNSs decorated silicone surface for their ability to prevent the biofilm growth. Similarly to the results shown above, the ACNSs on the surface of silicone were able to inhibit *E. coli* biofilm formation reducing the total mass of sessile bacteria up to 80 % when compared to the control epoxy treated silicone

1 (Figure 4 A). Furthermore, the fluorescence microscopy images after Live/Dead

2 staining corroborated this finding and well-established biofilm of live (stained in green)

3 and dead (stained in red) bacteria was visualized on the surface of the pristine silicone

4 material. In contrast, the functionalization with ACNSs resulted in significant reduction

5 of drug resistant *E. coli* biofilm and only few cells individually spread on the surface

6 were observed (Figure 4 B). At that stage the individual cells are considered less

7 virulent and even more susceptible to antibiotics at lower therapeutic dosages. Since the

8 concentration of NSs used to immobilize the silicone surface was previously found to be

9 innocuous towards fibroblasts cells [10], we believe the functionalization described

10 herein may constitute an efficient approach to obtain a biocompatible and anti-biofilm

11 surface for the prevention of *E. coli* associated infections and biofilms.

12

13 **Conclusions**

14 The inherent resistance of established biofilms to antibacterial drugs and their pervasive

15 involvement in implant-related infections has prompted the search for surfaces/coatings

16 that inhibit the initial stages of bacterial attachment, thus preventing or restricting the

17 occurrence of biofilm formation. Herein, highly antibacterial ACNSs were used to

18 develop permanent antibiofilm coatings on silicone material. The NSs were grafted onto

19 silicone surface using an epoxy/amine immobilization reaction. The ACNSs were found

20 to be efficient in preventing the *E. coli* biofilm growth after the covalent

21 immobilization. The sonochemically-induced nanospherization of cationic biopolymer

22 derivatives such as aminocellulose appears as a novel approach for improvement not

23 only of their bactericidal activity towards free *E. coli* cells, but also their inhibition

24 potential against biofilms. The further functionalization of surfaces with intact

nanospheres may provide catheters able to decrease the incidence of the *E. coli* associated infections and biofilms and significantly reduce hospital infections incidences coupled with increasing the usage time of the implants.

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1 **Scheme**s
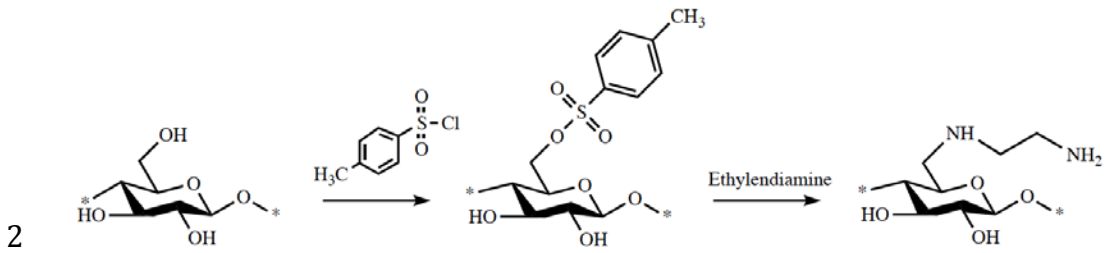
3 **Scheme 1.** Synthesis pathways for cellulose amination.

4

5
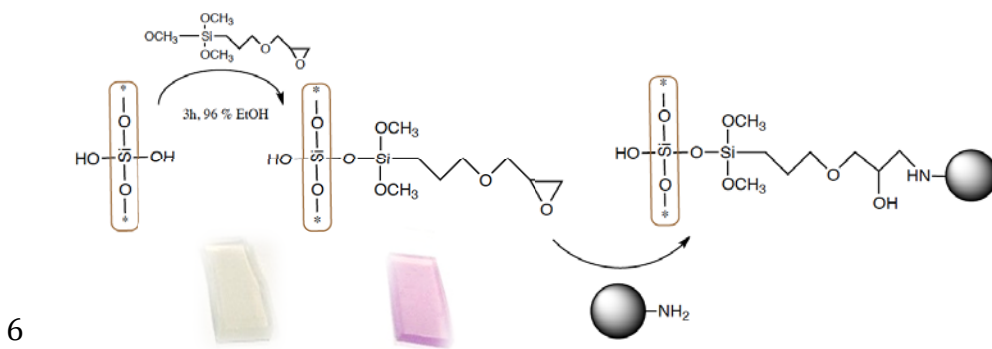
7 **Scheme 2.** Strategy used to functionalize silicone material: chemisorption of GOPTS

8 followed by the reaction of epoxy groups with ACNSs using the epoxy-amine curing

9 method and evaluation of the colour of silicone after reaction with p-NBP to assess the
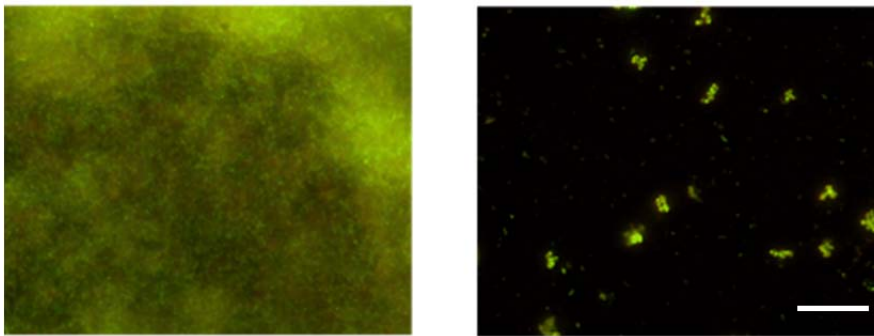
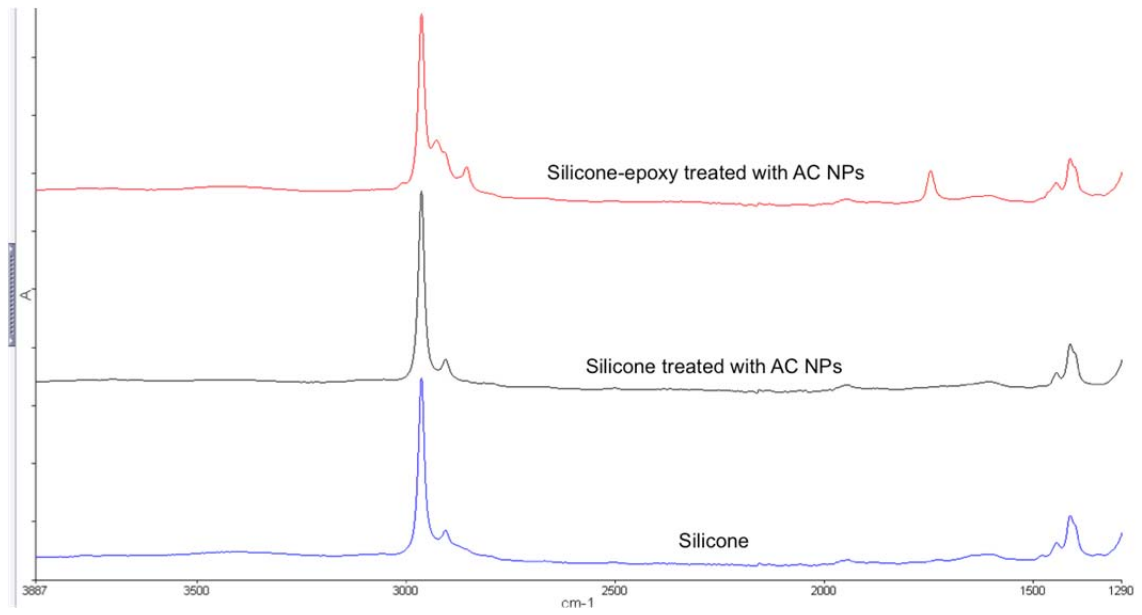10 efficiency of epoxy immobilization.

11

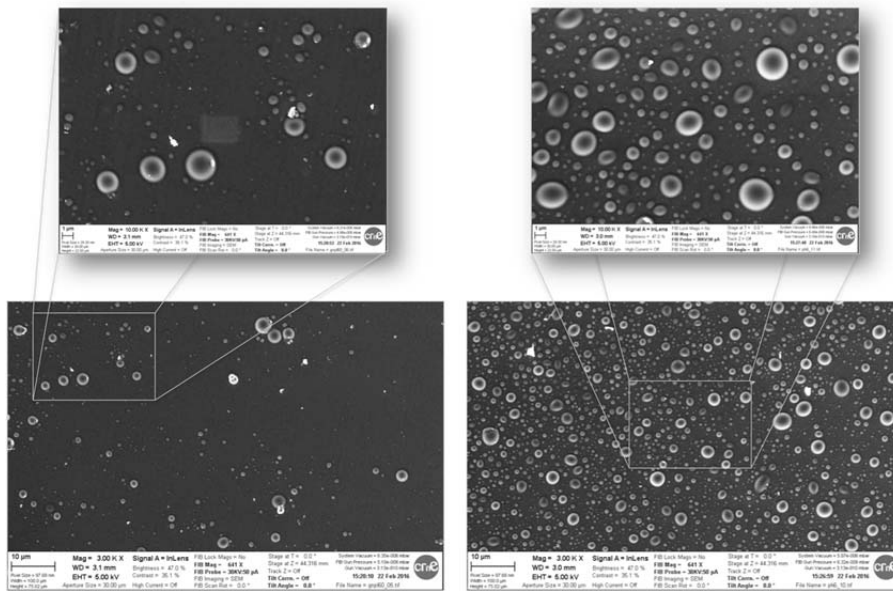1   **Figures**

2   A)
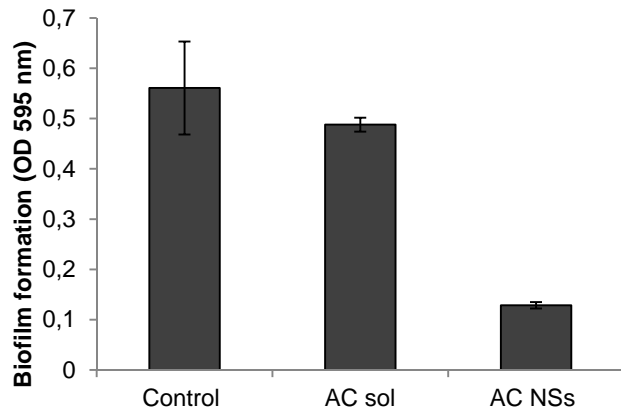
3

4   B)

5

6   **Figure 1**

7

1

2 **Figure 2**

3

Functionalization at pH 3   Functionalization at pH 6
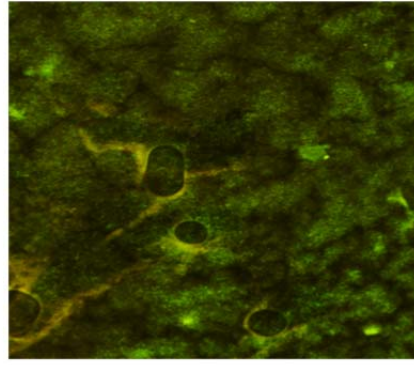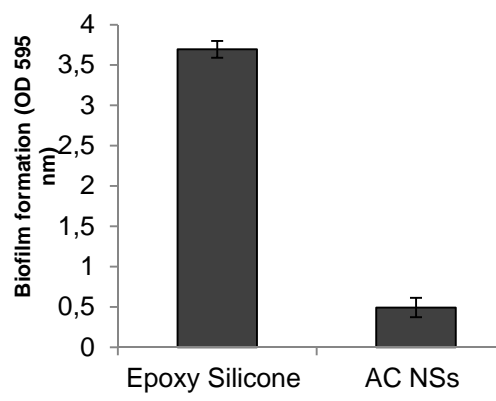
4

5 **Figure 3**

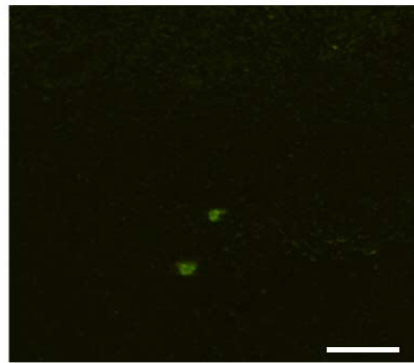6

A)   B)

Epoxy silicone

AC NSs decorated silicone

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2   **Figure 4**

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1   **Figure captions**

2

3   **Figure 1.** *E. coli* biofilm formation on polystyrene surface assessed by: A) crystal violet

4   assay (the OD_{595} is directly proportional to the amount of total biomass formed) and B)

5   Live/dead cells viability kit (ACsol left panel and AC NS on the right panel). The green

6   and red fluorescence are overlaid in for better comparison of live and dead cells. Scale

7   bar indicate 100 μm.

8   **Figure 2.** ATR-FTIR spectra of non-treated silicone (blue line), silicone (black line)

9   epoxy silicone treated with ACNSs at pH 6 (red line).

10   **Figure 3.** SEM images of ACNSs functionalized silicone surface.

11   **Figure 4.** *E. coli* biofilm formation on epoxy-treated silicone decorated with ACNSs

12   and unmodified silicone assessed by: A) crystal violet assay (the OD_{595} is directly

13   proportional to the amount of total biomass formed) and B) Live/dead cells viability kit.

14   The green and red fluorescence images are overlaid for better comparison of live and
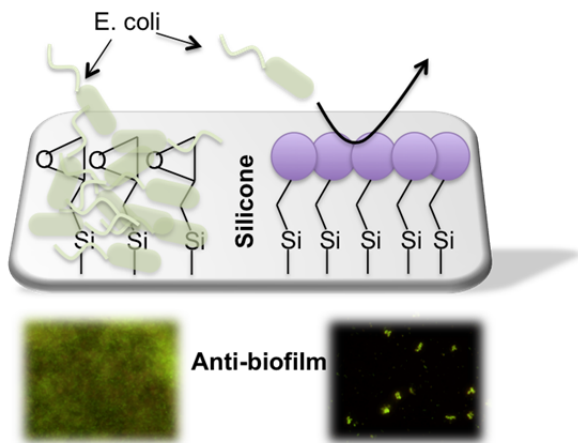
15   dead cells. Scale bar indicate 100 μm.

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1 **Graphical abstract**

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