Hierarchically patterned polyurethane microgrooves featuring nanopillars or nanoholes for neurite elongation and alignment

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Abstract
Surface micro- and nanostructures profoundly affect the functional performance of nerve regeneration implants by modulating neurite responses. However, few studies have investigated the impact of discrete nanostructures, such as nanopillars and nanoholes, and their combination with microgrooves on neurite outgrowth and alignment. Furthermore, numerous techniques have been developed for surface micro-/nanopatterning, but simple and low-cost approaches are quite limited. In this work, we show that nanopillars and nanoholes, and their combination with microgrooves, can be patterned on polyurethane (PU) films using a low-cost, reusable photosensitive master mold prepared via nanosphere lens lithography and UV-LED photolithography, with specific “reinforcement” methods for overcoming the inherent drawbacks of using photosensitive masters. We show that the PU nanopillars and nanoholes increase the neurite length of pheochromocytoma 12 (PC12) cells through unique growth cone interactions. Moreover, we demonstrate, for the first time, that hierarchically patterned nano-/microstructured PU films enhance both PC12 neurite elongation and alignment, showing the potential use of our proposed method for the micro-/nanopatterning of polymers for nerve tissue engineering.

Introduction
The surface features of biomaterials at the micro- and the nanoscale play a crucial role in modulating tissue responses and in determining the functional and temporal efficacy of implants [1]. Micro- and nanoscale surface structures affect cellular functions through micro- and nanometer-sized cell compartments, such as the nucleus, filopodia, and focal adhesions, resulting in
the modulation of signal cascades that leads to changes in cell proliferation, attachment, orientation, and differentiation, among others [2]. In nerve tissue engineering, the implant micro- and nanotopography serve as physical cues that promote nerve cell survival, neural stem cell recruitment and differentiation, and axonal guidance and regeneration [3]. The ability of topographical features to guide axons is particularly important in peripheral nerve regeneration, where unidirectional continuous micro-/nanostructures, such as fibers and grooves, in nerve guidance conduits facilitate axonal elongation and guidance and accelerate functional recovery [4].

Aside from continuous structures, different types of discrete micro- and nanostructures in the form of pillars, wires, tubes, holes, and cones have also been shown to positively affect neural functions and neurite outgrowth [3]. Studies on in vitro models for peripheral neurons show promising results for such structures, to wit, poly(3,4-ethylenedioxythiophene) nanotubes and SU-8 nanoholes resulted in significantly longer neurites in pheochromocytoma 12 (PC12) cells [5,6], poly(lactic-co-glycolic acid) nanodots enhanced the proliferation and neurite sprouting of Neuro-2a cells [7], and oriented elliptical Si microcones induced alignment and increased fasciculation in rat superior cervical ganglion axons [8]. With their effects complementing those of continuous structures, the question arises: Can discrete structures be combined with continuous structures for possible synergistic effects? Indeed, several studies have fabricated hierarchical discrete nanostructures on continuous microstructures in order to better mimic the micro- and the nanostructure of the native nerve microenvironment. While several of these focused on stem cell differentiation [9,10], a couple of studies explored their effects on axonal guidance. Lee et al. [11] found that nanorough microridges composed of laser-patterned Al/Al2O3 nanowires increase cell attachment and effectively guide dorsal root ganglia axons. Also, Huang et al. [12] showed that microgrooves with scattered nanodots result in neurite elongation and alignment on the hierarchically structured microgrooves; second, by demonstrating that the low-cost SU-8 substrates can be used as a reusable master mold to create nano-/micropatterns on polyurethane (PU), a soft, versatile material that has been used for nerve conduits [17]; and finally, by showing, for the first time, significant enhancement of both PC12 neurite elongation and alignment on the hierarchically structured microgrooves featuring nanopillars or nanoholes. Moreover, we found that replica molding using nano-/microstructured photoresist masters is a non-trivial step and requires specific “photoresist reinforcement” strategies to overcome inherent photoresist issues. Overall, our work demonstrates a promising method for the creation of hierarchical nano-/microstructures on various polymers for nerve implant applications.

Results and Discussion
Fabrication and characterization of PU nanopillar and nanohole substrates
We first fabricated nanopillar and nanohole arrays on medical-grade polyether-based PU (Tecothane®) to determine whether they have positive effects on PC12 neurite outgrowth. The fabrication process involves first creating the reusable photoresist master molds using NLL with a custom-made UV-LED exposure system [15,16]. This allows for the direct fabrication of a nanopillar array on an SU-8 film (Figure 1A(i) and (ii)). SU-8 generates a strong acid in UV-exposed areas, which, in turn, undergo acid-initiated crosslinking during the post-exposure baking step [18]. However, we found that the subsequent formation of a polydimethylsiloxane (PDMS) inverse mold from the SU-8 nanopillar array fails due to the breakage of the brittle SU-8 nanopillars (Supporting Information File 1, Other simple techniques, such as nanoimprinting and mold casting are ideal for pattern replication on thermoplastic and soluble polymers; however, they require master molds, which are typically fabricated using the abovementioned traditional techniques [14]. Therefore, there is still a need to develop simple and cost-effective fabrication methods applicable to a wide range of nano- and micropatterns and biomaterials.

In our previous studies, we have shown how nanosphere lens lithography (NLL) can be used with a low-cost ultraviolet light-emitting diode (UV-LED) system to create arrays of nanodots and nanopillars combined with microgrooves on the epoxy-based SU-8 negative photoresist [15,16]. While we found an improvement in PC12 neurite alignment on the ridge areas of nanopillared microgrooves, the overall alignment was not significantly different from that of plain microgrooves and there was a slight decrease in neurite length [16]. In this work, we provide significant advancements to our previous study in three main areas: first, by fabricating a new hierarchical SU-8 structure consisting of nanoholes on microgrooves; second, by demonstrating that the low-cost SU-8 substrates can be used as a reusable master mold to create nano-/micropatterns on polyurethane (PU), a soft, versatile material that has been used for nerve conduits [17]; and finally, by showing, for the first time, significant enhancement of both PC12 neurite elongation and alignment on the hierarchically structured microgrooves featuring nanopillars or nanoholes. Moreover, we found that replica molding using nano-/microstructured photoresist masters is a non-trivial step and requires specific “photoresist reinforcement” strategies to overcome inherent photoresist issues. Overall, our work demonstrates a promising method for the creation of hierarchical nano-/microstructures on various polymers for nerve implant applications.
Figure 1: Fabrication process and characteristics of the PU substrates (non-grooved). (A) Preparation of SU-8 nanopillar/hole master molds. (The steps enclosed by the blue dashed box are the “photoresist reinforcement” steps described in the main text.) (B) Replication of nanostructures on PU. (C–E) Scanning electron micrographs (SEM) of PU flat (C), nanopillar (D), and nanohole (E) substrates, with corresponding high-magnification images (insets). (Yellow arrowheads in (D) indicate smaller nanopillars. See further discussion in the text.) (F–H) Cross-sectional profile of the flat (F), nanopillar (G), and nanohole (H) PU surface from atomic force microscopy scans, showing the dimensions of the nanostructures (G, H). (Dimensions in parentheses were obtained from SEM images in Supporting Information File 1, Figure S4.) (I) Water contact angles on the PU films before and after O$_2$ plasma treatment (**p < 0.001; n = 6). (J–L) 3D confocal fluorescence micrographs of immunostained adsorbed laminin on PU flat (J), nanopillar (K), and nanohole (L) substrates.

Figure S1A,B), while further hard-baking to strengthen pillar adhesion causes SU-8 reflow, resulting in a dramatic decrease in the pillar aspect ratio (Supporting Information File 1, Figure S1C). Therefore, we employed a unique solution to “reinforce” the SU-8 nanopillars whereby the SU-8 is hard-baked while the nanostructures are encapsulated in cured PDMS (Figure 1A(iii)). This effectively entrapped the reflowing SU-8, allowing for the preservation of the nanopillar structures while...
further crosslinking the SU-8 (Figure 1A(iv)). This enabled the release of the PDMS film without breakage of the nanopillars (Supporting Information File 1, Figure S1D).

Application of NLL on the positive photoresist AZ1518 allowed for the formation of a nanohole array (Figure 1A(v)). AZ1518 contains a polymerized phenolic resin (Novolak) and a diazonaphthoquinone sulfonate photoactive chemical that is converted into a carboxylic acid upon UV exposure, resulting in increased solubility of the photoresist in the alkaline developer [19]. Nevertheless, the solvent of AZ1518 (and many other photoresists), propylene glycol monomethyl ether acetate, is also the main constituent of the SU-8 developer, rendering AZ1518 incompatible with the subsequent micropatterning step for the hierarchical structures. Therefore, we replicated the AZ1518 nanoholes unto SU-8 using capillary thermal imprinting and then crosslinked the imprinted SU-8 layer (Figure 1A(vi) and (vii)), resulting in a “reinforced” nanohole array (Figure 1A (viii)). Using these “reinforced” photoresist masters, PDMS replica molding and PU solvent casting allowed for the creation of the PU nanopillar and nanohole substrates (Figure 1B and Supporting Information File 1, Figure S2).

Scanning electron microscopy (SEM) images (Figure 1C–E) confirm the featureless surface of flat PU and the ordered arrays of nanopillars and nanoholes on the nanopatterned films. For the PU nanopillar substrate, some short pillars occasionally appeared (Figure 1D), which were also visible under an optical (metallurgical) microscope (Supporting Information File 1, Figure S3A). This was probably due to the polymerization of uncrosslinked PDMS in the mold nanoholes when the PDMS expands during PU casting (Supporting Information File 1, Figure S3B). When lots of short nanopillars had appeared, the PDMS mold was discarded and a new mold was prepared using the SU-8 master. In contrast, we did not observe shallow PU nanoholes on our samples, which was probably due to the more open surface of the PDMS mold nanopillars, resulting in a lesser degree of contact among uncrosslinked PDMS monomers (Supporting Information File 1, Figure S3C).

Atomic force microscopy (AFM) scans of the samples (Figure 1F–H) show that the nanopillars and nanoholes have sub-micrometer feature sizes and a periodicity of around 1.2 μm. Due to AFM measurement artifacts, especially for lateral dimensions of high-aspect-ratio nanostructures [16], we re-measured some dimensions using SEM images (Supporting Information File 1, Figure S4). The nanopillars were around 860 nm high and 330 nm wide at the base and had a rounded tip. The nanoholes were around 960 nm deep and 860 nm wide at the opening and had a rounded bottom. The space between the pillars and holes were around 860 nm and 330 nm, respectively.

The wettability of a surface is a good predictor of protein adsorption and bioactivity [20]. For the extracellular matrix protein laminin, good adsorption and cell growth have been found on hydrophilic, O₂ plasma-treated substrates [21]. We measured the water contact angle (CA) on the PU samples (Figure 1I) and found that all of the as-fabricated samples were hydrophobic (CA > 90°), with the nanostructured substrates being more so. Since the CA for flat PU indicates a hydrophobic surface, the increase in CA on the nanopillar and nanohole substrates may be due to either a Wenzel- or a Cassie-type of wetting [22]. To improve wetting on the substrates, we treated our samples with mild O₂ plasma before laminin incubation. After plasma treatment, all samples became hydrophilic (CA < 80°), with the nanopillar substrate having the smallest CAs (CA ≈ 30°). Based on confocal fluorescence microscopy of immunostained samples (Figure 1J–L), laminin successfully adsorbed onto the O₂ plasma-treated PU samples. There was good laminin coverage on all of the samples, even on the nanostructures, as indicated by the fluorescence patterns conforming to the structure shapes. Laminin was also successfully coated on the flat areas surrounding the pillars and holes, as shown in the corresponding confocal slices in Supporting Information File 1, Figure S5. O₂ plasma treatment of PU enables strong laminin adsorption possibly due to the introduction of C=O and C–OH bonds on the surface, which leads to a negative charge for electrostatic attraction of positively charged laminin molecules [23,24].

**PC12 neurite outgrowth on PU nanopillars and nanoholes**

PC12 cells attached well on all the laminin-coated substrates and showed good viability and proliferation on the nanostructured PU films, especially on the PU nanoholes (Supporting Information File 1, Figure S6). After differentiation, PC12 cells extended beta-III tubulin positive neurites, with longer neurites appearing on the nanostructured substrates (Figure 2A–C and Supporting Information File 1, Figure S7) and more short projections emanating from the soma on the flat substrate (white arrowheads). Quantification of neurite length (Figure 2D) confirms increased neurite length per cell on the nanopillar and nanohole substrates, with means at least 1.2× higher than that of flat PU (p < 0.05). Analysis of the neurite branch lengths (Figure 2E) also shows that the proportion of branches greater than or equal to 40 μm is bigger on the nanopatterned substrates compared with flat, while the proportion of branches less than 20 μm (i.e., the average soma diameter) is smaller (Supporting Information File 1, Figure S8). Considering only branches greater than or equal to 20 μm, the improvement in neurite length is enhanced, with the median

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neurite length per cell on the nanopatterned substrates at least 1.6× greater than that of flat PU ($p < 0.01$) (Figure 2F). We did not find any differences in the number of primary neurites per cell among the substrates (Supporting Information File 1, Figure S9A), and the neurite length normalized to the primary neurite count showed similar enhancement on the nanopatterned substrates (Supporting Information File 1, Figure S9B,C). There were also no significant differences in the amount of neurite branching among the nanopatterned substrates and the flat PU (Supporting Information File 1, Figure S10).

The number of cells and primary neurites was lower on the nanopillar substrate compared with flat PU (Supporting Information File 1, Figure S11), probably because of lower proliferation of differentiating PC12 cells on the nanopillars. Correlational analysis shows that the cell and primary neurite counts have a moderate to strong inverse relationship with the neurite length parameters (Supporting Information File 1, Table S1) and thus might have contributed to the increased normalized neurite length on the nanopillar substrate. However, we think that they were not the determining factors as neurites were indeed qualitatively longer on the nanopillar substrate (Figure 2B), and sufficient space was available on the flat substrate for neurite extension despite higher cell and primary neurite counts (Figure 2A).

SEM images of the PC12 cells on PU samples (Figure 2G–I) show that the neurites grew unimpeded on both the flat and nanostructured substrates. On the nanopillar array (Figure 2H), the neurites and neurite tips (presumably growth cones; insets) layed on the flat base area between the pillars and most likely anchored on most of the nanopillar sidewalls, resulting in a more complex, meta-2D or “2.5D” growth environment [25]. (Note that the SEM shows collapsed nanopillars due to the drying process.) Growth cone filopodia also appear to extend
towards nanopillars on the flat base areas. On the nanohole array (Figure 2I), the neurites and growth cones passed between and over the holes. However, the growth cone filopodia passed along the spaces and edges between the holes without being suspended across the holes.

The enhancement of PC12 neurite elongation observed here is similar to that previously reported on SU-8 nanoholes (around 250 nm in diameter and 50 nm in spacing) [6] and different from that on gold nanopillars (around 230 nm in diameter and 70 nm in spacing) and nanopores (around 200 nm in diameter and 40 nm in spacing) [26]. Furthermore, in our previous study [16], SU-8 nanopillars with slightly smaller inter-pillar gaps (around 750 nm) than those used here inhibited neurite outgrowth. Thus, it appears that PC12 neurites elongate when continuous narrow pathways of sufficient width are present, as in the nanohole array here and in [6], as well as when the neurites can grow on a flat surface with multiple attachment areas, as in the nanopillar array here (neurites growing on the base area) and as opposed to that in our previous study (neurites growing on top of the pillars) [16] and in [26]. We hypothesize that the nanopillar sidewalls and nanohole spaces act as attachment and guidance cues, respectively, for the growth cone filopodia, facilitating its forward movement and the extension of the neurite, as illustrated in Figure 2J. It will be interesting to determine if such cues provided by the nanopillars and nanoholes could be modulated by the nanopillar/nanohole spacings. This could be the subject of future work utilizing nanosphere lenses with different sizes.

**Fabrication and characterization of PU pillar–groove and hole–groove substrates**

After confirming that PU nanopillars and nanoholes promote neurite elongation in PC12 cells, we combined the nanostructures with microgrooves to determine their potential use in neurite guidance. Following the strategy in our previous work [16], hierarchical structures in the SU-8 master were achieved using a simple UV-LED photolithography step following NLL (Figure 3A(i) and (ii)). The starting samples for photolithography were a hard-baked SU-8 film, SU-8 nanopillars (not hard-baked), and SU-8 nanoholes for the creation of plain microgrooves (“microgroove”), nanopillars on microgrooves (“pillar–groove”), and nanoholes on microgrooves (“hole–groove”), respectively. (Note that for the nanoholes, the spin-coating of the second SU-8 layer occasionally results in a patchy film, probably because of some residual PDMS from the nanopillar imprinter. Investigation of the SU-8 post-exposure baking parameters during thermal imprinting seems necessary for more consistent results in the future.) As with the SU-8 nanopillar array, a “reinforcement” step consisting of hard-baking in cured PDMS (Figure 3A(iii)) was necessary for the pillar–groove master to prevent breakage of the nanopillars (Figure 3A(iv)).

After PDMS replica molding and PU solvent casting (Figure 3B), the hierarchical patterns were successfully replicated on PU films (Figure 3C–E and Supporting Information File 1, Figure S12). As can be seen from the AFM scans (Figure 3F–H), the microridge areas were around 15 µm in width, while the microgroove areas were slightly wider, around 20–24 µm. The microridge height was 1.4–1.5 µm on the microgroove and hole–groove substrates, while it was 1.1 µm or 2 µm on the pillar–groove substrate if measured to the tip or base of the nanopillars, respectively.

**Water CA measurements on the grooved PU samples lead to two distinct CAs on each sample: one with the CA baseline in parallel with the groove axis (CA [∥]; Figure 3I) and another with the CA baseline perpendicular to the groove axis (CA [⊥]; Figure 3J). As can be seen in Figure 3I, the CA [∥] values have similar trends as their non-grooved counterparts (Figure 1I), indicating dominance of the nanostructure properties in this orientation. In contrast, the CA [⊥] values were higher and remained quite close to each other in magnitude even after O2 plasma treatment, signifying the dominance of the microgroove properties in this orientation. Despite the relatively high CA [⊥] values after plasma treatment, the lower CA [∥] values (<60°) indicate that the surface was hydrophilic enough along the groove direction, and good solution coverage could be achieved during laminin coating.

We also confirmed laminin adsorption on the grooved PU samples using confocal fluorescence microscopy. Figure 3K–M show successful laminin coating of the samples on both the microridge and microgroove areas, with brighter groove areas on the pillar–groove and hole–groove samples (Figure 3L and M) due to the higher effective surface area resulting from the nanostructures.

**PC12 neurite outgrowth on nanopatterned PU microgrooves**

PC12 cells also attached well on all grooved PU substrates and showed good viability and proliferation on the hierarchically patterned microgrooves, especially on the hole–groove substrate (Supporting Information File 1, Figure S13). After differentiation, PC12 cells extended neurites preferentially in the direction of the microgrooves (Figure 4A–C and Supporting Information File 1, Figure S14). Quantification of neurite extension shows that only the pillar-groove substrate yielded a statistically different distribution of neurite length per cell compared with the microgroove substrate (p = 0.049), with a mean around 1.2× higher (p = 0.008) (Figure 4D). Nevertheless, there was...
Figure 3: Fabrication process and characteristics of the grooved PU substrates. (A) Preparation of SU-8 nanopillar/hole on microgroove master molds. (The step enclosed by the blue dashed box is the “photosist reinforcement” step described in the main text.) (B) Replication of nano-micro-structures on PU. (C–E) Scanning electron micrographs of PU microgroove (C), pillar–groove (D), and hole–groove (E) substrates, with corresponding high-magnification images (insets). (F–H) Cross-sectional profile of the microgroove (F), pillar–groove (G), and hole–groove (H) PU surface from atomic force microscopy scans, showing the dimensions of the structures. (I, J) Water contact angles on the PU films before and after O₂ plasma treatment with the contact angle baseline in parallel with (I) or perpendicular to (J) the groove axis (**p < 0.001; n = 6). (K–M) 3D confocal fluorescence micrographs of immunostained adsorbed laminin on PU microgroove (K), pillar–groove (L), and hole–groove (M) substrates.

still a larger proportion of longer neurite branches (≥20 µm) and a smaller proportion of branches less than 20 µm on both the pillar–groove and hole–groove substrates than on the microgroove substrate (Figure 4E and Supporting Information File 1, Figure S15). Considering only branches greater than or equal to 20 µm, the improvement in neurite length was again enhanced, with the mean neurite length per cell on the nanopatterned substrates at least 1.5× greater than that of the microgroove substrate (p < 0.001) (Figure 4F). There were no significant differences in the number of primary neurites per cell among the substrates (Supporting Information File 1, Figure S16A), and a similar enhancement of the neurite length on nanopatterned substrates was found when normalized to the primary neurite count (Supporting Information File 1, Figure S16B,C). Moreover,
Figure 4: Characteristics of PC12 neurite outgrowth on grooved PU substrates. (A–C) Fluorescence micrographs (merged) of PC12 cells with stained actin, beta-III tubulin, and nucleus on PU microgroove (A), pillar–groove (B), and hole–groove (C) substrates. (White arrowheads indicate short projections, while white double-headed arrows indicate the direction of the grooves. Background subtraction and brightness/contrast adjustment were performed. For separate, non-background-subtracted images, please refer to Supporting Information File 1, Figure S14.) (D–I) Quantification of different PC12 neurite parameters: (D) total neurite length per cell; (E) relative frequency histogram of neurite branch length; (F) total neurite length of branches greater than or equal to 20 µm per cell; (G) relative amount of aligned neurites in entire area; (H) relative amount of aligned neurites on groove and ridge areas; and (I) percentage of neurite length on groove areas (\( * \ p < 0.05; ** \ @ \ p < 0.01; *** \ p < 0.001; n = 15 \)). In (D), the microgroove and pillar–groove substrates have normally distributed data (with the means shown as black-filled squares). The asterisk (*) and at (@) symbols in (D) refer to the distribution and mean, respectively. (J) Hypothesized behavior of PC12 growth cones on the PU microgroove (i), pillar–groove (ii), and hole–groove (iii) substrates.

there were also no significant differences in the amount of neurite branching among the substrates (Supporting Information File 1, Figure S17). Similar to the non-grooved substrate, the pillar–groove substrate had lower cell and primary neurite counts (Supporting Information File 1, Figure S18), and moderate to strong negative correlations were found between the cell and primary neurite counts and neurite length parameters (Supporting Information File 1, Table S2), which might have slightly inflated the normalized neurite length values for the pillar–groove substrate.

Quantitative analysis of neurite directionality on the grooved substrates reveals an improvement in neurite alignment on the nanopatterned grooves, as can be seen in the increase in the amount of neurites aligned within 15° of the grooves, considering all neurite branches (Figure 4G and Supporting Information File 1, Figure S19A) and branches greater than or equal to 20 µm only (Supporting Information File 1, Figure S19B). In particular, the amount of aligned neurites was at least 1.17x higher on the nanopatterned substrates compared to the plain microgrooves (\( p < 0.01 \)). Further analysis of the neurite directionality on the ridge and groove areas (Supporting Information File 1, Figure S20) reveals that the enhancement of neurite alignment on the pillar–groove and hole–groove substrates was due to the improvement in alignment on the ridge areas for the former and on groove areas for the latter (Figure 4H). More-
over, the improvement in neurite alignment on the ridges of the pillar–groove substrate was accompanied by an overall decrease in neurite localization on the grooves (i.e., an increase in neurites on ridges); however, no increase in neurite localization on the grooves in the hole–groove substrate was observed (Figure 4I). This change in neurite localization seems not to be mainly caused by the location of the soma as only an increase in soma localization on the groove areas for the hole–groove substrate was found (Supporting Information File 1, Figure S21).

The alignment of neurites on microgrooves has been mainly attributed to the bending rigidity of the neurite cytoskeleton, leading to the resistance of the growth cone to cross groove steps (Figure 4J(i)) [27,28]. Here, we observe that the microgrooves with a width of around 20 µm and a depth of around 1.4–2 µm are sufficient to elicit good alignment of PC12 neurites, consistent with previous studies [29,30]. Meanwhile, the improved neurite alignment on the pillar–groove substrate was probably due to the failure of the growth cone filopodia to establish stable adhesions on the highly discontinuous pillar tips [16], resulting in the retraction of the growth cone (Figure 4J(ii)) and the increased confinement of the neurites on the ridge areas. Although the ridges were slightly higher on the pillar–groove substrate (when measured from the pillar base), this was probably not the main determining factor since no improved alignment on the groove areas was observed. The enhanced neurite alignment on the hole–groove substrate could be due to the guidance effect of the submicrometer-wide spaces between the holes on the growth cone filopodia, amplifying the guidance effect of the steps on the groove areas (Figure 4J(iii)).

**Conclusion**

We have developed a low-cost method for the creation of nanopillar or nanohole arrays and hierarchical structures consisting of nanopillar/holes on microgrooves on PU films for the enhancement of neurite elongation and alignment. The fabrication process involves the use of NLL and UV-LED photolithography for master mold preparation and soft lithography and solvent casting for PU film patterning. Challenges in the use of photoresist master molds for PDMS replica molding and microgroove formation were addressed using “reinforcement” strategies. Differentiation of PC12 cells on the PU substrates resulted in longer neurites on the nanopillar and nanohole arrays. Furthermore, when combined with microgrooves, the discrete nanostructures enhanced not only neurite elongation but also neurite alignment as compared with a plain microgrooved PU substrate. The low-cost method presented in this study facilitates the creation of nano-/microstructures on substrates of different solvent-castable polymers without the use of expensive equipment. Moreover, the hierarchically patterned microgrooves featuring nanopillars and nanoholes provide an additional strategy for the enhancement of next-generation nerve guidance conduits.

**Experimental Materials**

Polystyrene nanospheres (ca. 1.1 µm diameter), laminin from Engelbreth–Helm–Swarm murine sarcoma, and nerve growth factor (2.5S, from murine submaxillary glands) were purchased from Sigma, Merck KGaA (Germany). SU-8 50 and SU-8 developer were obtained from Kayaku Advanced Materials (MA, USA), while AZ1518 and AZ 300 MIF developer were purchased from MicroChemicals GmbH (Germany). PDMS, γ-butyrolactone (GBL), and PU pellets (Tecothane®, clear; TT-1085A) were obtained from Sil-More Industrial Ltd. (Taiwan), Echo Chemical Co., Ltd. (Taiwan), and Lubrizol Advanced Materials, Inc. (OH, USA), respectively. Dimethylacetamide (Alfa Aesar), phosphate-buffered saline (PBS), sera, rhodamine–phalloidin (RP), 4′,6-diamidino-2-phenylindole (DAPI), and Alexa Fluor 488–beta-III tubulin antibody (AF488-anti-β3 tubulin) were obtained from Thermo Fisher Scientific (MA, USA). RPMI 1640 medium, sodium pyruvate, and HEPES buffer were purchased from Corning (NY, USA). The reusable polystyrene nanosphere lens array (1.1 µm) embedded in PDMS for UV-light focusing was prepared according to procedures described in our previous study [16].

**Preparation of nano-/micropatterned SU-8 master molds**

The formation of the SU-8 nanopillar array was similar to that of our previous study [16]. Briefly, a thin SU-8 layer was first hard-baked on a 2.5 cm × 2.5 cm glass slide as an adhesion layer. Then, the SU-8 layer to be patterned was spun-coated using a GBL-diluted SU-8 solution (SU-8 50/GBL vol. ratio 1:0.7) at 5000 rpm and soft-baked at 95 °C for 160 s. An array of polystyrene nanospheres (1.1 µm) embedded in PDMS was placed in conformal contact with the SU-8, and then exposure was performed at a dose of 35–42 mJ·cm⁻² (Figure 1A(i)). (Older PS-NS/PDMS films seem to require slightly higher UV doses, maybe because of UV oxidation of the films or changes in the shape of the PS-NS caused by residual GBL in the SU-8.) The SU-8 was subjected to a post-exposure bake at 95 °C for 2 min, followed by development in the SU-8 developer for 1 min, rinsing with isopropyl alcohol, and N₂ drying (Figure 1A(ii)). To prevent breakage of the SU-8 nanopillars during PDMS molding, a SU-8 “reinforcement” step was performed, which entailed hard-baking the photoresist with an encapsulating cured PDMS (A/B wt. ratio 15:1) (Figure 1A(iii)). The hard-baking process was as follows: 65 °C for 5 min, 95 °C for 5 min, 150 °C for 15 min, 170 °C for 1 h, and 195 °C for 1 h. Afterwards, the sample was allowed to
cool down to room temperature (RT), and then the PDMS was peeled off (Figure 1A(iv)).

To form the “reinforced” SU-8 nanohole mold, a nanohole template was first formed on the positive photoresist AZ1518, which served as a template for creating a PDMS nanopillar structure for the capillary thermal imprinting of SU-8. An AZ1518 film was spin-coated on glass coverslips at 5000 rpm and soft-baked at 100 °C for 1.5 min. Exposure was performed at a dose of 13 mJ·cm\(^{-2}\) with an array of 1.1 µm polystyrene nanospheres (in PDMS) in conformal contact with the photoresist (Figure 1A(i)). AZ1518 was developed with AZ 300 MIF developer for 30 s, followed by rinsing with ultrapure water and N\(_2\) drying (Figure 1A(v)). The nanopatterned AZ1518 was finally post-baked at 120 °C for 2 min to improve substrate adhesion. PDMS (A/B wt. ratio 10:1) was poured over the nanopatterned AZ1518, degassed, cured at 65 °C on a hotplate overnight, and peeled off to obtain PDMS nanopillars (Figure 1A(vi)).

For imprinting nanoholes on SU-8, a diluted SU-8 solution (SU-8 50/GBL vol. ratio 1:0.37) was spin-coated on a 2.5 cm × 2.5 cm glass slide at 5000 rpm and soft-baked at 95 °C for 2 min. The PDMS nanopillar array was placed in conformal contact with the SU-8, and then the sample was baked at 95 °C for 5 min for thermal reflow of the SU-8 (Figure 1A(vii)). The sample was allowed to cool down to RT and then was flood-exposed at a dose of 180 mJ·cm\(^{-2}\). Afterwards, the sample was subjected to post-exposure bake and hard bake via a stepwise increase in temperature: 95 °C for 3 min, 150 °C for 15 min, and 165 °C for 1 h. After baking, the sample was allowed to cool down to RT, and then the PDMS nanopillar film was peeled off (Figure 1A(viii)).

The fabrication process for the grooved SU-8 molds was similar to that of our previous study (Figure 3A(i) and (ii)) [16]. Briefly, the starting samples were 2.5 cm × 2.5 cm glass slides with a hard-baked SU-8 film, SU-8 nanopillars (not hard-baked), and SU-8 nanoholes for the creation of SU-8 micro-groove, pillar–groove, and hole–groove substrates, respectively. For the SU-8 microgroove and pillar–groove samples, a SU-8 50/GBL volume ratio of 1:0.37 was used with the following processing parameters: spin speed of 5000 rpm, soft bake at 95 °C for 3 min, UV dose of 200 mJ·cm\(^{-2}\), post-exposure bake at 95 °C for 2.5 min, and development for 60 s and 70 s for microgroove and pillar–groove, respectively. A less dilute SU-8 solution with SU-8 50/GBL volume ratio of 1:0.33 was used for the hole–groove sample to create microgrooves of similar depth with the following adjusted parameters: soft bake at 95 °C for 190 s, UV dose of 210 mJ·cm\(^{-2}\), post-exposure bake at 95 °C for 160 s, and development for 80 s. The SU-8 pillar–groove sample was also subjected to a hard-baking “reinforcement” step similar to that of the SU-8 nanopillars, as described above (Figure 3A(iii) and (iv)).

All photoresist exposure steps were performed using a custom-made UV-LED system [15], while all baking steps were done on a hotplate.

**Preparation of nano-/micropatterned PU films**

PDMS inverse molds were prepared using the SU-8 master molds by pouring PDMS (A/B wt. ratio 10:1) onto the molds, degassing, and curing at 65 °C on a hotplate overnight (Figure 1B(i) and (ii), Figure 3B(i) and (ii)). A flat PDMS mold was also prepared using the top side of a cured PDMS sheet. PDMS rings were placed on the molds to create a well to hold the PU solution during casting.

The PU samples were prepared by solvent casting onto the PDMS molds (Figure 1B(iii) and 3B(iii)). To facilitate filling of the nano- and microstructures, a dilute 5 wt % PU solution in dimethylacetamide was first cast into the molds twice, with slow, partial drying at 80 °C in an oven for 10 min and 15 min in the first and second casting, respectively. Then, to increase the sample thickness for easier handling, a less dilute 10 wt % PU was cast over the partially dried PU, slowly dried in the oven at 80 °C for 10 min, and then fully dried in the oven at 100 °C for 3 h. Afterwards, the samples were allowed to cool down to RT, and then the PU films were carefully peeled off from the molds and cut to form the final samples (Figure 1B(iv) and 3B(iv)).

The PU substrates were characterized using SEM, AFM, and water CA measurements, as described in detail in Supporting Information File 1.

**PC12 culture and neurite outgrowth experiment**

PC12 cells (ATCC CRL-1721) were cultured in RPMI 1640 medium (with l-glutamine and sodium bicarbonate), supplemented with HEPES buffer (25 mM), sodium pyruvate (1 mM), heat-inactivated horse serum (10% v/v), fetal bovine serum (5% v/v), and penicillin/streptomycin (100 U·mL\(^{-1}\)/100 µg·mL\(^{-1}\)) in a humidified CO\(_2\) incubator (37 °C, 5% CO\(_2\)).

The PC12 neurite outgrowth experiment was divided into two parts: The first part was neurite outgrowth on PU flat, nanopillar, and nanohole substrates; the second part was neurite outgrowth on PU microgroove, pillar–groove, and hole–groove substrates. The samples were treated with O\(_2\) plasma (30 W, 20 sccm O\(_2\), 30 s) to make the PU surface hydrophilic and enhance the adsorption of laminin. The samples were then placed...
in a 24-well culture plate, sterilized with UV in a biosafety cabinet for 1 h, and coated with 10 µg·mL⁻¹ laminin in Ca²⁺/Mg²⁺-free PBS (1 mL per well) at 4 °C overnight. After coating, the substrates were washed twice with Ca²⁺/Mg²⁺-free PBS.

PC12 cells were seeded on the PU samples at 13 × 10³ cells per well. After overnight incubation in growth medium, the medium in each well was replaced with 1 mL differentiation medium, which was composed of RPMI 1640 medium (with L-glutamine and sodium bicarbonate), supplemented with HEPES buffer (25 mM), sodium pyruvate (1 mM), heat-inactivated horse serum (1% v/v), penicillin/streptomycin (100 U·mL⁻¹/100 µg·mL⁻¹), and nerve growth factor (100 ng·mL⁻¹). The cells were differentiated for six days, with half of the medium being replaced with fresh differentiation medium every two days. The neurite outgrowth experiments were performed in triplicate.

The adsorbed laminin on the PU substrates was observed using confocal fluorescence microscopy. PC12 neurite outgrowth after differentiation was characterized using fluorescence micrographs of cells stained with RP, AF488-anti-β3 tubulin, and DAPI. Quantification of neurite parameters were performed using a semi-automatic method described in our previous study [16]. SEM was also performed to observe the neurite morphology. Further details on the characterization procedures and statistical analyses can be found in Supporting Information File 1.

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Supporting Information

Supporting Information File 1
Additional details of experimental methods and supplementary data.
[https://www.beilstein-journals.org/bjnano/content/supplementary/2190-4286-14-96-S1.pdf]


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Fluorescent bioinspired albumin/polydopamine nanoparticles and their interactions with Escherichia coli cells

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Abstract

Inspired by the eumelanin aggregates in human skin, polydopamine nanoparticles (PDA NPs) are promising nanovectors for biomedical applications, especially because of their biocompatibility. We synthesized and characterized fluorescent PDA NPs of 10–25 nm diameter based on a protein containing a lysine–glutamate diad (bovine serum albumin, BSA) and determined whether they can penetrate and accumulate in bacterial cells to serve as a marker or drug nanocarrier. Three fluorescent PDA NPs were designed to allow for tracking in three different wavelength ranges by oxidizing BSA/PDA NPs (Ox-BSA/PDA NPs) or labelling with fluorescein 5-isothiocyanate (FITC-BSA/PDA NPs) or rhodamine B isothiocyanate (RhBITC-BSA/PDA NPs). FITC-BSA/PDA NPs and RhBITC-BSA/PDA NPs penetrated and accumulated in both cell wall and inner compartments of Escherichia coli (E. coli) cells. The fluorescence signals were diffuse or displayed aggregate-like patterns with both labelled NPs and free dyes. RhBITC-BSA/PDA NPs led to the most intense fluorescence in cells. Penetration and accumulation of NPs was not accompanied by a bactericidal or inhibitory effect of growth as demonstrated with the Gram-negative E. coli species and confirmed with a Gram-positive bacterial species (Staphylococcus aureus). Altogether, these results allow us to envisage the use of labelled BSA/PDA NPs to track bacteria and carry drugs in the core of bacterial cells.

Introduction

Organic nanoparticles (ONPs) are used to target and deliver drugs to tissue and eukaryotic cells [1,2], or bacteria and biofilms [3,4]. As nanovectors of drugs, they can deliver drugs locally, leading to a more efficient drug activity. Also, the required doses and the drug impact on healthy tissues compared to the free drug are lowered. Regarding the dramatic emer-
ence and spreading of antimicrobial resistance of bacteria [5], this appears as a promising route to deliver antimicrobials while reducing the drug doses and subsequent harmful side effects in antibacterial applications. To this end, different types of ONPs have been used, such as liposomes [6] and nanoparticles (NPs) of poly(lactic-co-glycolic acid) (PLGA) [7], polycaprolactone [8], and chitosan [9]. Furthermore, fluorescent ONPs are a promising way to facilitate the localization of NPs in cells through fluorescence imaging. They can also be used for fluorescent labelling of cells, especially for live cell imaging, provided that the ONPs are harmless for cells. This has been developed for eukaryotic cells [10], but the use of ONPs for labelling bacterial cells is still rare and not described in literature for alive bacterial cells. The main limitation is probably the frequent cytotoxic effect of ONPs on bacteria.

Inspired by the eumelanin aggregates in human skin, polydopamine nanoaggregates (here referred to as nanoparticles, i.e., PDA NPs) have emerged as promising nanovectors for biomedical applications [11,12], especially because of their biocompatibility [13,14] and photothermic properties [15,16]. These properties can even be controlled by an external signal [17-19]. PDA NPs are formed upon oxidation in dopamine (DA) solutions with additives such as surfactants, polyelectrolytes, and proteins [14]. These properties can even be controlled by an external signal [17-19]. PDA NPs are formed upon oxidation in dopamine (DA) solutions with additives such as surfactants, polyelectrolytes, and proteins [14]. Eumelanin-like NPs with a diameter less than 20 nm have been obtained by this method. The additive plays a crucial role in the control of the NP size. Specifically, Bergtold et al. demonstrated that a protein (e.g. chromofungin) containing a diad of lysine (K) and glutamate (E) (Figure 1a,b) in its sequence allows for the control of the formation of PDA NPs, in contrast to an additive without a KE diad (e.g., catestatin) [13]. During the formation process, hydroxy groups of dopamine form hydrogen bonds with carboxylic groups (COO\(^{-}\)) of glutamate (pK\(_{a}\) = 4.3), whereas protonated amino groups (NH\(_{3}^{+}\)) of lysine (pK\(_{a}\) = 10.5) further stabilize the aggregate by cation–π interactions with the aromatic ring of dopamine (Figure 1c). For this, K and E residues must be next to each other. Even a single glycine residue (G) located between K and E can destabilize the aggregates [13]. Among such possible additives, the albumin protein is an interesting candidate since it contains one KE diad and is already widely used in biology. Its hydrodynamic radius of about 4 nm at pH 7 makes it possible to envisage NP sizes close to 10 nm [20]. Chassepot and Ball prepared eumelanin-like particles in the presence of albumin, whose sizes decreased with the amount of protein down to 30 nm in diameter [14]. The structure of these albumin/PDA NPs has not been elucidated completely. It has been demonstrated that proteins are present in the NPs’ shell; they might also be present in the core (Figure 1d). Nevertheless, their potential both for fluorescent labelling of alive bacterial cells and as nanovector for antibacterial activity is high because of their small size and because any antibacterial natural or synthetic peptide containing KE diads may be used to create such PDA NPs. Fluorescent PDA NPs made with a KE diad-contain-
The ability of the NPs to pass through the cell membrane was shown to allow for the modification of their fluorescence properties. This also modified the outer surface chemistry; thus, the modified proteins come into contact with already formed PDA. Bergtold et al. proposed that the size control is exerted by the specific interaction of the KE diad with DA [13]. Most of the proteins can penetrate and accumulate in bacterial cells [22], thus suggesting that other ONPs may have a similar fate in bacteria. In general, the mechanisms of action of ONPs used as drug nanocarriers in antibacterial applications are expected to vary with the nanoparticle type (e.g., liposomes or PLGA NPs) but have not been elucidated so far. In contrast to inorganic NPs [23,24], it is unclear whether ONPs can penetrate bacterial cells. Alipour et al. have shown that a 170 nm diameter liposomal nanocarrier increased the accumulation of an antibiotic (polymyxin B) in Gram-negative bacterial cells, but the penetration of lipopolysaccharides into the cell was not proved [25]. In general, organic nanocarriers are often reported to penetrate mammalian cells infected by bacteria, improving the drug accumulation in these eukaryotic cells and increasing the antibacterial efficiency of the drug [3,4,9,26]. However, the nanocarriers were not found in the bacterial cells, and the question was rarely mentioned at all. Thus, whether the increase in effectiveness of antibiotics carried by NPs is the result of the penetration of the complete nanocarrier–drug system into bacteria or rather an effect of the destabilization of the bacterial cell membrane by interactions with the nanocarriers (thus allowing for the penetration of the drug into the bacteria) is not known (Figure 1e). Yet, the accumulation of ONPs in bacterial cells is crucial if ONPs are to be used for fluorescent labelling of cells. Also, in the case of nanocarrier–drug systems, it may increase the dose of drug delivered close to the bacterial cell machinery and, therefore, improve the treatment efficacy. If the photothermic properties of the ONPs are to be exploited, the efficacy of the antibacterial treatment may also completely depend on the capacity of ONPs to penetrate and accumulate in the bacterial cell. Therefore, the fate of PDA NPs related to bacterial cells is a crucial aspect for their further use in antibacterial applications.

The objective of the study was to synthesize fluorescent PDA NPs based on a lysine–glutamate-diad containing protein and to determine whether they can enter and accumulate in bacterial cells. The investigation has been conducted with NPs made of polydopamine (PDA) and bovine serum albumin (BSA), and Escherichia coli (E. coli) bacteria as a bacterial model. Three synthesis routes were used to prepare these fluorescent BSA/PDA NPs, based either on the oxidation of pristine BSA/PDA NPs (Ox-BSA/PDA NPs) or on labelling with fluorescent 5-isothiocyanate (FITC) or rhodamine B isothiocyanate (RhBITC) fluorescent dyes (FITC-BSA/PDA NPs and RhBITC-BSA/PDA NPs, respectively). According to Ma et al., Ox-BSA/PDA NPs are expected to emit a maximum of fluorescence in the 450–500 nm range under 405 nm light irradiation [21]. FITC-BSA/PDA NPs and RhBITC-BSA/PDA NPs are expected to emit similarly to free FITC (emission in the green range if excited at 488 nm) and RhBITC (emission in the red range if excited at 561 nm), respectively.

**Results and Discussion**

Pristine BSA/PDA NPs with BSA/DA ratios ranging from 0.25 to 10 and three different types of fluorescent BSA/PDA NPs with a BSA/DA ratio of 10 were synthesized and characterized. Three synthesis routes were used to prepare these fluorescent BSA/PDA NPs, based either on the oxidation of pristine BSA/PDA NPs (Ox-BSA/PDA NPs) or on labelling with fluorescent 5-isothiocyanate (FITC) or rhodamine B isothiocyanate (RhBITC) fluorescent dyes (FITC-BSA/PDA NPs and RhBITC-BSA/PDA NPs, respectively). According to Ma et al., Ox-BSA/PDA NPs are expected to emit a maximum of fluorescence in the 450–500 nm range under 405 nm light irradiation [21]. FITC-BSA/PDA NPs and RhBITC-BSA/PDA NPs are expected to emit similarly to free FITC (emission in the green range if excited at 488 nm) and RhBITC (emission in the red range if excited at 561 nm), respectively.

BSA/PDA NPs can be as small as 10 nm

**Synthesis of stable BSA/PDA NPs with size control**

Pristine polydopamine nanoparticles (BSA/PDA NPs) were prepared according to Bergtold et al. [13] (Figure 2a–c). BSA and DA solutions were mixed in Tris buffer with ratios varying from 0.25 to 10. Contrary to a DA solution in Tris buffer, the mixtures of BSA and DA in Tris buffer inhibited the deposition of a PDA film on the wall of the reaction beakers (Figure 3a). This suggested that BSA/PDA aggregates formed and that almost all the free DA molecules were consumed in these aggregates, as already mentioned by Bergtold and co-workers [13].

A series of pristine BSA/PDA NPs with a mean diameter in number ranging from 66 ± 16 to 9 ± 3 nm was obtained (Figure 3b; see Supporting Information File 1, Table S1 for mean diameter in intensity) by increasing BSA/DA ratio from 0.25 to 10. The formation of BSA/PDA NPs is attributed to the interaction between DA and BSA, which contains the KE diad and allows for the control of the NP formation [13]. During the synthesis, DA is added into the BSA solution, thus avoiding that the proteins come into contact with already formed PDA. Bergtold et al. proposed that the size control is exerted by the specific interaction of the KE diad with DA [13]. Most of the DA molecules interact with the proteins, thus limiting the binding of proteins with each other or with PDA aggregates.
This results in an increase in aggregate (i.e., particle) size. Hence, a rapid decrease in diameter was observed for BSA/DA ratios from 0.25 to 3. However, a plateau value was reached above a ratio of 6, leading to BSA/PDA NPs of 11 ± 3 nm and 9 ± 3 nm in diameter for BSA/DA ratios of 6 and 10, respectively. The size of the BSA protein is probably the limiting factor to a further decrease of the NP size. Indeed, one BSA molecule has an hydrodynamic diameter of 3–4 nm at physiological pH [20]. The resulting number \( N \) of nanoparticles per milliliter and self-polymerization reaction yield \( \eta \) obtained with BSA/DA ratios of...
Figure 3: Solutions and sizes of pristine and fluorescent BSA/PDA NPs. (a) Evidence of the inhibition of a PDA film deposition on the wall of the reaction beaker of (left) BSA/PDA solution in Tris buffer compared to (right) PDA solution in Tris buffer. (b) Mean hydrodynamic diameter of pristine BSA/PDA NPs as a function of the BSA/DA ratio. (c) Photograph of pristine BSA/PDA NPs after two years of storage. (d) Mean hydrodynamic diameter in number of pristine PDA/BSA-NPs depending on the solvent. (e) Mean hydrodynamic diameter in number of pristine PDA/BSA-NPs depending on the pH value. (f) Mean hydrodynamic diameter in number of pristine BSA/PDA NPs, Ox-BSA/PDA NPs, FITC-BSA/PDA NPs, and RhBITC-BSA/PDA NPs (all synthesized with a BSA/DA ratio of 10).

3 and 10 were calculated with Equation 1 and Equation 2, respectively:

\[ N = \frac{m_{\text{dry}}}{V} \times \frac{1}{\frac{4}{3} \pi r^3 \rho_{\text{PDA}}} \]  \hspace{1cm} (1)

\[ \eta = \frac{C_f}{C_f^{\text{DA}} + C_i^{\text{BSA}}} \]  \hspace{1cm} (2)

where \( V \) is the volume of the NP solution remainder after freeze-drying, \( m_{\text{dry}} \) is the mass of the NP foam measured after freeze-drying, and \( \rho_{\text{PDA}} \) is the density of polydopamine (1.52 g·cm\(^{-3}\)) [27]. Final concentration and number of BSA/PDA NPs increased by increasing the BSA/DA ratio from 3 and 10 (\( 2 \times 10^{14} \) and...
4 × 10^{15}\text{NPs/mL}, respectively), indicating a similar reaction yield of 85% (Supporting Information File 1, Table S2). The dispersion was changed by changing the ratio of BSA, but the reaction yield was maintained. This suggests that BSA acted as a “knife” changing the size, but not the composition, of the nanoparticles.

The suspension of BSA/PDA NPs was stable over two years of storage in Tris buffer in the dark at ambient temperature, as shown by the good dispersion and the absence of precipitates and deposition on the container wall (Figure 3c). Good stability and dispersion were maintained after dialysis with a 100 kDa cut-off membrane, which allowed for the removal of free BSA molecules. As shown by Chassepot and Ball [14] and Bergtold et al. [13], human serum albumin and other proteins play an important role in size control and stability of PDA NPs. Similarly, the stability of BSA/PDA NPs is probably due to BSA thanks to the strong PDA/KE interactions reported by Bergtold et al. This is supported by the progressive inhibition of the deposition of a PDA film on the wall of the reaction beaker, which was mentioned above and observed above a certain amount of BSA (Figure 3a). This allowed us to assume that BSA molecules below a critical amount cannot surround all DA molecules (some DA molecules thus form a thin film of PDA on the beaker’s wall), whereas they stabilize PDA NPs above this amount.

The suspension stability was maintained when BSA/PDA NPs were in suspension in H_{2}O or 150 mM NaCl solution. However, the mean hydrodynamic diameter varied with the solvent, with an increase by up to 50% for a BSA/DA ratio of 3 and 60% for a BSA/DA ratio of 10. The smallest BSA/PDA NPs had a mean diameter of 9 nm in 50 mM Tris and of 10 nm in H_{2}O, but of 18 nm in 150 mM NaCl solution (Figure 3d). This increase was attributed to two co-existing phenomena related to the high salt concentration in 150 mM NaCl. First, the high ionic strength of 150 mM NaCl decreased long-range effect and intensity of electrostatic interactions compared to the other solvents, thus favoring other types of interactions (such as attractive van der Waals interactions) and, therefore, aggregation. Second, the increase in salt concentration might have led to an increase in the Stern layer thickness, resulting in a larger hydrodynamic diameter measured by DLS.

The size of BSA/PDA NPs remained stable as a function of pH at acidic pH, but increased moderately under alkaline conditions (Figure 3e). Chen et al. reported that PDA NPs deteriorated above pH 11 with, first, a decrease in size before the NP morphology changed into nanosheets [28]. However, in the case of BSA/PDA NPs, the NPs size increased when the pH value was above 6.5. In addition, precipitation, sedimentation, or degradation of the dispersion were not observed, showing that the suspension stability was unchanged. These results suggest that BSA prevented the degradation in alkaline solution, probably through heavily stabilizing the NPs.

The BSA/PDA NPs suspensions were freeze-dried to obtain dried NPs that could easily be stored and re-suspended at the needed concentration for further experiments. During the freeze-drying process, BSA/PDA NPs acted as surfactant and were located around water droplets when the water was rapidly sublimated, which resulted in the formation of a foam (Supporting Information File 1, Figure S1a). After freeze-drying, the foam could be easily re-suspended in Milli-Q® water, resulting in a well-dispersed black suspension (Supporting Information File 1, Figure S1b). However, in the case of a BSA/DA ratio of 0.25, a powder was obtained instead of a foam (Supporting Information File 1, Figure S1c). This was probably due to a lack of BSA molecules to surround every molecule of PDA, which may have favored bridges involving BSA between NPs and further formation of aggregates (Supporting Information File 1, Figure S1d). This powder could not be re-dissolved in water, NaCl, or Tris buffer. Furthermore, the mean hydrodynamic diameter slightly increased for both BSA/DA ratios of 3 and 10 after dialysis and freeze-drying (22% and 18%, respectively) (Supporting Information File 1, Figure S1e). During water evaporation, NPs may have been brought closer, leading to the formation of irreversible interactions and slight aggregation. This only slight increase in size was considered insignificant regarding the possible effect on the interactions of NPs with bacteria; hence, freeze-drying was further used for the storage of BSA/PDA NPs.

**Fluorescent modifications of BSA/PDA NPs**

BSA/PDA NPs were modified to produce fluorescent BSA/PDA NPs by three different methods, that is, the oxidation of pristine BSA/PDA NPs (Ox-BSA/PDA NPs) (Figure 2d) or the labelling of BSA with FITC or RhBITC fluorescent dyes (FITC-BSA/PDA NPs and RhBITC-BSA/PDA NPs, respectively) (Figure 2e). All fluorescent BSA/PDA NPs were made from pristine BSA/PDA NPs synthesized with a BSA/DA ratio of 10. This resulted in Ox-BSA/PDA NPs and FITC-BSA/PDA NPs with a mean diameter similar to that of pristine BSA/PDA NPs and RhBITC-BSA/PDA NPs of about 20 nm in diameter (Figure 3f). A small decrease in size of Ox-BSA/PDA NPs was expected and measured (30% decrease), as also noticed by Ma et al., probably due to the degradation of the pristine BSA/PDA NPs upon oxidation [21]. However, regarding the size values and deviations measured, the decrease was considered insignificant here. The increase in size of RhBITC-BSA/PDA NPs upon labelling with RhBITC can be explained by the simultaneous presence of a positive charge and two additional diethylamino...
groups in RhBITC, which are absent in FITC. These two additional chemical moieties may have induced some weak aggregation between the negatively charged RhBITC-BSA/PDA NPs, in contrast to FITC-BSA/PDA NPs.

Ox-BSA/PDA NPs were produced by the oxidation of BSA/PDA NPs for 24 h with H$_2$O$_2$. The BSA/PDA NP suspension, initially dark brown, turned to translucent light brown during oxidation (Supporting Information File 1, Figure S2b). Under UV light, the Ox-BSA/PDA NPs suspension emitted fluorescence, in contrast to the suspension before oxidation (Supporting Information File 1, Figure S2c). This may result from the conjugation and electronic density changes induced during the reaction with H$_2$O$_2$, during which hydroxy groups were oxidized into ketones. Eventually, the large majority of polydopamine functional groups are expected to be ketones with only a few remaining of hydroxy groups (Supporting Information File 1, Figure S2a). In addition, labelling of pristine BSA/PDA NPs with FITC and RhBITC dyes led to an obvious change in color of the suspension, from brown (Supporting Information File 1, Figure S3a) to yellow and red (Supporting Information File 1, Figure S3b,c), respectively, in agreement with the color of the corresponding dye.

Pristine BSA/PDA-NPs exhibited a wide absorption spectrum with a maximum of absorption at 280 nm, whereas Ox-PDA/BSA-NPs showed a less broad and intense absorption with a maximum at 285 nm (Figure 4a). In other words, the oxidation leads to a bathochromic (increase in wavelength) shift due to an increase in conjugation when –OH groups were oxidized into =O groups. Moreover, a hypochromic (decrease in absorption intensity) effect was measured, which may have resulted from the degradation of the BSA/PDA-NPs upon oxidation (suggested by the insignificant decrease in size of Ox-BSA/PDA NPs compared to pristine BSA/PDA NPs) and a reduction of concentration or molar extinction coefficient during the prolonged oxidation. Indeed, it is well known that the oxidation of indole quinone groups in melanin-like materials leads to the...
release of pyrrole carboxylic acids [29]. In addition, Ox-BSA/PDA NPs revealed an emission spectrum centered around 490 nm. It is to note that no significant shift of the maximum emission wavelength depending on the excitation wavelength was measured (Supporting Information File 1, Figure S4a), contrary to what has been mentioned in the literature [21,30]. This effect is not elucidated so far, but it is important to note that the NPs considered here are different from those of Zhang and Ma, since PDA is here associated to BSA. Moreover, the Stokes shift of Ox-BSA/PDA NPs (\(\lambda_{\text{max,ems}} - \lambda_{\text{max,abs}}\)) is estimated to be about 200 nm (Supporting Information File 1, Figure S4b), which is higher than that of common fluorochromes such as RhBITC and FITC (about 25 nm) [31] or even DAPI (about 100 nm) [32]. This big difference in energy is consistent with the ability of PDA to heat up under irradiation by non-radiative relaxation. This is favorable to minimize the superposition between absorption and emission peaks, but might lead to a low quantum yield and, thus, poor emission intensity. Indeed, the quantum yield of Ox-BSA/PDA NPs in water (\(\Phi\)) was calculated to be 0.1% with Equation 3, using DAPI in water as the reference [33].

\[
\Phi = \frac{\Phi_{\text{DAPI}} \times \frac{\lambda_{\text{Ox-BSA/PDA NPs}}}{\lambda_{\text{DAPI}}}}{\Phi_{\text{DAPI}} \times \frac{\eta_{\text{Ox-BSA/PDA NPs}}^2}{\eta_{\text{DAPI}}} - \frac{\eta_{\text{DAPI}}}{\eta_{\text{Ox-BSA/PDA NPs}}}},
\]  

(3)

where, \(\Phi_{\text{DAPI}}\) is the fluorescence quantum yield of DAPI when unbound to DNA (4%) [32], \(\lambda_{\text{Ox-BSA/PDA NPs}}\) and \(\lambda_{\text{DAPI}}\) are the integrated fluorescence intensities of the light emitted by Ox-BSA/PDA NPs and DAPI, respectively, \(\lambda_{\text{Ox-BSA/PDA NPs}}\) and \(\lambda_{\text{DAPI}}\) are the absorption values of Ox-BSA/PDA NPs and DAPI solutions, respectively, and \(\eta_{\text{Ox-BSA/PDA NPs}}\) and \(\eta_{\text{DAPI}}\) are the refractive indices of the solvents of Ox-BSA/PDA NPs and DAPI solutions, respectively. Because the solvent is the same (water) for both Ox-BSA/PDA NPs and DAPI solutions, the value of \(\frac{\eta_{\text{Ox-BSA/PDA NPs}}^2}{\eta_{\text{DAPI}}}\) is here equal to 1.

Compared to the quantum yields of FITC, RhBITC, and DAPI bound to DNA, which are in the range of 40–90% at room temperature [31,32,34], Ox-BSA/PDA NPs reveal low emission efficiency. However, even if the quantum yield of Ox-BSA/PDA NPs is low, it is significantly higher than the quantum yield of non-oxidized NPs, which are not fluorescent at all [35].

FITC- and RhBITC-BSA/PDA NPs exhibited absorption maxima at 500 and 560 nm, respectively, shifted by 8 nm compared with free FITC and RhBITC (absorption maxima at 492 and 552 nm, respectively) (Figure 4c,d and Supporting Information File 1, Figure S5). This probably results from a change in polarity in the close environment of the bound fluorophore. In addition, the absorption intensity of FITC-BSA/PDA and RhBITC-BSA/PDA NPs above 600 nm is a typical feature of melanin materials. The high background (decreasing from 400 to 800 nm and similar to the absorption spectrum of pristine BSA/PDA NPs) was attributed to the absorption by NPs (Figure 4a). The emission spectra of FITC- and RhBITC-BSA/PDA NPs were centered around 520 and 578 nm, respectively, which is similar to free FITC and RhBITC (emission maxima at 519 and 578 nm, respectively) under excitation at 488 and 550 nm, respectively (Figure 4c,d and Supporting Information File 1, Figure S5). Unfortunately, because PDA absorbs a significant part of the light emitted by the fluorophores, the intensity cannot be related to the number of FITC- and RhBITC-labelled NPs. However, the absorption intensities were used to estimate (even though roughly) the equivalent quantities of free FITC and RhBITC to be used as controls in the further bacteriological assays. The estimated equivalent quantities corresponding to a concentration of fluorescent NPs of 2 mg/mL (164 µM for FITC and 373 µM for RhBITC) were calculated as described in the Experimental section.

The stability of the fluorescent labelling was tested after four months of ageing of FITC- and RhBITC-BSA/PDA NPs. Dialysis was performed on the four months old NP suspensions to retrieve FITC or RhBITC molecules possibly released from the labelled NPs by following the same procedure as described below for the elimination of unbound labelling molecules (see Experimental section). The fraction of released dye was estimated on the basis of the emission intensity of the dialysate and the initial quantity of labelled NPs (Supporting Information File 1, Figure S6). The fractions were less than 1% (1.5 µM for FITC and 0.5 µM of RhBITC) of the initial dye quantity, showing the high stability of the labelling and of the labelled NPs.

FITC-BSA/PDA NPs and RhBITC-BSA/PDA NPs tend to accumulate in E. coli cells with heterogeneous patterns

Whether pristine and fluorescent BSA/PDA NPs can interact with bacterial cells (especially E. coli) is unknown. Therefore, the possible accumulation of pristine and fluorescent BSA/PDA NPs on and in cells was studied with the smallest NPs (about 10 nm diameter) since NP penetration in cells is expected to be inversely related to NP size [36].
tine BSA/PDA NPs were not detected at excitation and emission wavelengths corresponding to FITC and RhBITC dyes. It was also impossible to identify the specific fluorescent signal emitted by Ox-BSA/PDA NPs in absence of an adequate laser for the excitation of Ox-BSA/PDA NPs at 285 nm. Under excitation with a higher wavelength (405 nm), a low fluorescence signal was detected in the 460–541 nm and 415–482 nm ranges with the standard and the high-resolution microscope, respectively (Supporting Information File 1, Figure S7a). In part, it may result from the fluorescence of Ox-BSA/PDA NPs at \( \lambda_{exc} = 405 \) nm; however, it is more probably due to the intrinsic fluorescence of bacteria. Indeed, \textit{E. coli} cells alone and \textit{E. coli} cells cultivated with free FITC or RhBITC, or FITC- or RhBITC-BSA/PDA NPs revealed a similar fluorescence signal when excited at 405 nm (Supporting Information File 1, Figure S7a). This can be attributed to the intrinsic fluorescence of bacteria under 405 nm excitation due to porphyrins [37,38]. With Ox-BSA/PDA NPs, no significant difference in fluorescence intensity could be detected in this emission range under 405 nm excitation compared to the intrinsic fluorescence background. This prevents a conclusion on whether Ox-BSA/PDA NPs may have accumulated on or in \textit{E. coli} cells.

Under excitation at 488 nm, cells cultivated with FITC-BSA/PDA NPs and free FITC emitted slight fluorescence in the 495–634 nm and 496–565 nm ranges (observed with standard and high-resolution microscopes, respectively) (Figure 5a, Supporting Information File 1, Figure S7c), in contrast to \textit{E. coli} cells alone or cultivated with RhBITC-BSA/PDA NPs or free RhBITC. Under excitation at 561 nm, \textit{E. coli} cells cultivated with RhBITC-BSA/PDA NPs or free RhBITC emitted fluorescence in the 567–703 nm and 553–628 nm ranges (observed with standard and high-resolution microscopes, respectively) (Figure 5a, Supporting Information File 1, Figure S7). These emission ranges are in agreement with the fluorescence characteristics of FITC-BSA/PDA NPs and RhBITC-BSA/PDA NPs (Figure 4c,d) and can, thus, be attributed to FITC or RhBITC. Hence, FITC alone, RhBITC alone, FITC-BSA/PDA NPs, and RhBITC-BSA/PDA NPs were all able to label \textit{E. coli} cells. However, a much higher intensity was observed with RhBITC and RhBITC-BSA/PDA NPs than in the other experiments as confirmed by quantifying the maximal fluorescence measured in the micrographs (Figure 5b). This may result from the positive charges carried by RhBITC at physiological pH, which are absent in the FITC molecules. The positive charges are expected to participate in attractive electrostatic interactions with the negatively charged bacterial membrane, that is, the phosphates of their phospholipids. This facilitates the intimate contact between RhBITC-BSA/PDA NPs and the bacterial membrane and, possibly, the subsequent penetration of the NPs.

The localization of the fluorescence emission related to bacterial cells was determined on the basis of micrographs extracted from 3D-stack images obtained with the high-resolution confocal laser scanning microscope (CLSM, Stellaris 5, Leica Biosystems, Wetzlar, Germany). In all experiments with dyes (FITC-BSA/PDA NPs, RhBITC-BSA/PDA NPs, free FITC, and free RhBITC in equivalent concentrations), the fluorescence signal related to RhBITC or FITC was emitted at the location of the cells (Figure 5a,c and Figure 6). The fluorescence intensity in cells incubated with RhBITC-BSA/PDA NPs was about 15% of the intensity in cells incubated with the equivalent quantity of free RhBITC (Figure 5b). It reached about 100% with FITC-BSA/PDA NPs compared to free FITC. These ratios are much higher than expected if only free dye molecules released from NPs had penetrated the cells (1% as estimated above). Hence, they prove that the fluorescence measured in cells was not emitted by free molecules of dye but by molecules bound to NPs.

More specifically, the fluorescence appeared to come from the cell wall and from some inner parts of the bacterial cells with a frequent location at the poles of the cell. The fluorescence profiles confirm (i) that dyes accumulate in the cell wall rather than on the outside as shown by the co-localization of the intrinsic (blue) and dye-related fluorescence signals (dashed lines and black arrows in Figure 6b, Supporting Information File 1, Figure S8) and (ii) that the fluorescence was present as clusters in inner cell compartments (empty arrows in Figure 6b, Supporting Information File 1, Figure S8). This accumulation of labelled BSA/PDA NPs may be related to proteins since fluorescent dyes such as RhBITC can interact with proteins in a non-covalent manner [39]. In addition, proteins can aggregate at the poles or in the cytosol of \textit{E. coli} (and other bacterial species) during cell division or as the result of cellular ageing or under external stressors [40,41]. This may match the distribution patterns observed with FITC- and RhBITC-BSA/PDA NPs. Moreover, by themselves or by the excitation light used to reveal them, the labelled NPs may be the external stressor leading to cell ageing and subsequent protein aggregation as proposed by Rang and co-workers [42]. In any case, our results indicate that FITC- and RhBITC-BSA/PDA NPs were able to penetrate and accumulate in the cell wall and internal compartments of \textit{E. coli} cells in significant quantities.

BSA/PDA NPs are prone to damage bacterial cells since they can penetrate them. The possible harmful impact on bacterial growth was therefore tested with \textit{E. coli} and \textit{S. aureus}. However, we showed that BSA/PDA NPs in concentrations corresponding to more than \( 10^7 \) NPs per bacterial cell (0.2–2.0 mg/mL; \( 4 \times 10^{13} \) to \( 4 \times 10^{14} \) NPs/mL) were unable to significantly modify the growth of a \textit{E. coli} population.
Figure 5: Fluorescence characterization of *E. coli* cultures with FITC-BSA/PDA NPs, free FITC, RhBITC-BSA/PDA NPs, free RhBITC, or alone (NPs synthesized with a BSA/DA ratio of 10). (a) Typical fluorescence micrographs extracted from 3D-stack images measured with a high-resolution CLSM (Stellaris 5, Leica Biosystems, Wetzlar, Germany) (λ<sub>exc</sub> of 405, 488, or 543 nm; range of λ<sub>em</sub> of 415–482, 496–565, and 553–628 nm, respectively). (b) Maximal fluorescence intensity measured in bacterial cells for each condition (mean ± SD of all the micrographs of each condition). (c) Mean fluorescence intensity of bacterial cells for each condition (mean counts in bacterial cells per field) (mean ± SD of all complete bacteria in all micrographs of each condition).

(Figure 6a). This was maintained regardless of the surface properties of the NPs since pristine, Ox-, and RhBITC-BSA/PDA NPs all failed to inhibit *E. coli* growth (Figure 7b). It can be noted that the population growth was even slightly but significantly favored in the presence of pristine and fluorescent BSA/PDA NPs. This effect, which is not elucidated so far, was also observed regarding the growth of *S. aureus* populations (Supporting Information File 1, Figure S9). Especially the absence of *E. coli* growth inhibition by RhBITC-BSA/PDA NPs allows us to envisage the use of the labelled BSA/PDA NPs developed in this study to track bacterial cells, but also to carry drugs in bacterial cells thanks to their penetration capacity.
Conclusion

Fluorescent nanoparticles of PDA with about 15 nm diameter were synthesized with BSA as a KE diad-carrying protein. Blue fluorescence was obtained under 405 nm excitation by oxidation of pristine BSA/PDA NPs, while green fluorescence under 488 nm illumination and red fluorescence under 543 nm illumination were achieved by labelling pristine BSA/PDA NPs with fluorescent FITC and RhBITC dyes, respectively. The fluorescent NPs did not significantly vary in size compared to pristine NPs, with diameters in the range of 10–15 nm for Ox-BSA/PDA NPs and FITC-BSA/PDA NPs and a slightly higher diameter for RhBITC-BSA/PDA NPs (ca. 20 nm). The suspensions of pristine and fluorescent NPs were all stable over months, allowing for long-term storage. The capacity of FITC- and RhBITC-BSA/PDA NPs to penetrate bacterial cell walls, to enter the cell core, and to accumulate in the different compartments was demonstrated for E. coli cells. Both FITC- and RhBITC-BSA/PDA NPs, as well as free FITC and RhBITC dyes, exhibited fluorescence in aggregate-like patterns in the cells. However, the growth of bacteria was not inhibited, which
allows us to envisage fluorescent tracking of live bacterial cells using these fluorescent NPs. The capacity of these NPs to penetrate cell walls might also enable their use to carry drugs in the core of bacterial cells.

**Experimental**

**Synthesis**

**Reagents**

Dopamine (DA) hydrochloride and bovine serum albumin (BSA) were purchased from Merck (Darmstadt, Germany) and used without purification. Fluorescein isothiocyanate (FITC), rhodamine B isothiocyanate (RhBITC), hydrochloric acid 37% (wt/v), absolute ethanol and Hellmanex® were purchased from Merck (Darmstadt, Germany). Tris(hydroxymethyl)aminomethane (Tris buffer) was purchased from Euromedex (Strasbourg, France). 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Fisher Scientific (Illkirch, France). All aqueous solutions were made using Milli-Q® water (ρ = 18.2 MΩ·cm; Millipore® Reverse Osmosis system; Merck, Darmstadt, Germany).

**BSA/polydopamine nanoparticles**

Polydopamine nanoparticles (BSA/PDA NPs) were prepared according to Bergtold and co-workers [13] (Figure 2a–c). Tris buffer solution was prepared by dissolution of 6.055 g (5 × 10^-2 mol) of Tris powder in 1 L of Milli-Q® water. The pH was adjusted to 8.5 by adding hydrochloric acid. X mg of BSA (X depending on the chosen BSA/DA wt/wt ratio; see Table 1) were slowly dissolved in 100 mL Tris buffer to avoid foam formation. 200 mg of dopamine hydrochloride was added to yield a solution of 2 mg/mL of DA. This solution was gently shaken at ambient temperature in the dark. After 24 h, a homogenous black suspension of BSA/PDA NPs was obtained. Small oligomers, unreacted dopamine monomers, and Tris salt were removed from the reaction medium by dialysis. 20 mL of the suspension were dialyzed against 2 L of Milli-Q® water using a Spectra/Por® dialysis membrane with a molecular weight cutoff of 8–10 kDa (purchased from Fisher Scientific S.A.S., Illkirch, France). The dialysis was performed in the dark and followed by spectroscopic measurements at 280 nm (absorption of dopamine) to check for the absence of oligomers in the
dialysate at the end of the dialysis process [13]. Dialysis was repeated three times, yielding a total dilution of the dialysate by a factor of $10^6$. The solution was frozen at $-80 \, ^\circ C$ for 1 h, and water was eliminated by freeze-drying (Alpha 1-4 LDplus; Christ, Osterode am Harz, Germany) for 48 h to provide lyophilized foam, which was stored at room temperature and protected from light before use.

<table>
<thead>
<tr>
<th>Table 1: Weight of BSA (X mg) and dopamine (DA), and the corresponding BSA/DA ratio used to synthesize the BSA/PDA NPs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA/DA ratio</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>0.25</td>
</tr>
<tr>
<td>1.00</td>
</tr>
<tr>
<td>3.00</td>
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<tr>
<td>6.00</td>
</tr>
<tr>
<td>10.00</td>
</tr>
</tbody>
</table>

Fluorescent BSA/polydopamine nanoparticles

Fluorescent BSA/PDA nanoparticles with a BSA/DA ratio of 10 were synthesized by using two different ways: (i) by oxidation of pristine BSA/PDA nanoparticles and (ii) by adding fluorescent labels to the BSA used to prepare BSA/PDA nanoparticles (Figure 2d–e).

**PDA oxidation.** Fluorescent, oxidized polydopamine nanoparticles (Ox-PDA/BSA NPs) were prepared on the basis of pristine BSA/PDA NPs synthesized as described above with a BSA/DA ratio of 10 ($1.1 \times 10^{-6}$ mol BSA, 1 equiv). Pristine BSA/PDA NPs were then modified by a procedure inspired from Ma et al. [21] as follows: A suspension of BSA/PDA NPs foam was prepared in Milli-Q® water at a concentration of 10 mg BSA/PDA NPs per milliliter; 7.5 mL of this suspension (pH 6.8) and 2 mL of H$_2$O$_2$ solution (30 wt %) were stirred at room temperature in the dark for 1 h and followed by a dialysis against 1 L of Milli-Q® water using a Spectra/Por® dialysis membrane with a molecular weight cut-off at 8–10 kDa to eliminate unbound labelling molecules. The dialysis was done in the dark and repeated three times yielding a total dilution of the dialysate by a factor of $10^6$. The evolution of the dialysis was followed by measuring the absorption spectrum of the dialysate. The same procedure was used to prepare RhBITC-BSA/PDA NPs, except that FITC was replaced by RhBITC. The concentrated solution of RhBITC (8.25 mM) was prepared by dissolving 4.4 mg in 1 mL of DMSO.

**Characterization of the BSA/polydopamine nanoparticles**

**Size measurement**

The diameter of the NPs was measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS from Malvern Panalytical (Malvern, UK). Measurements were performed while taking into account a refractive index of 1.73 + 0.02i for PDA (the imaginary part corresponds to the absorption coefficient) at a wavelength of 589 nm, that is, close to the wavelength of the laser used in the device (633 nm). Samples were diluted to get an absorption below 0.1. Size distribution results are given in intensity and can be expressed also in volume or number. Standard deviations of the sample’s mean hydrodynamic diameters as well as the polydispersity index are provided in Supporting Information File 1, Table S1. In this study, results given in number will be used.

**Characterization of the fluorescence properties**

Fluorescence spectra were recorded with a spectrophotometer-fluorimeter SAFAS Xenius XM 529 (SAFAS Monaco, Monaco, Monaco). Light absorption of BSA/PDA NPs, Ox-BSA/PDA NPs, FITC-BSA/PDA NPs, and RhBITC-BSA/PDA NPs was measured between 200 and 800 nm against Tris buffer. The fluorescence of Ox-BSA/PDA NPs, FITC-BSA/PDA NPs, and RhBITC-BSA/PDA NPs was characterized by emission spectra measurements at different excitation wavelengths in the range of 350–650 nm (photomultiplier voltage from 600 to 1000 V specified in the figure captions if needed; step of 1 or 5 nm). The emission intensity was compared with that of the fluorescent dyes, that is, FITC for FITC-BSA/PDA NPs and the fluorescent probes were linked through the isothiocyanate groups of FITC and RhBITC and the amine group of BSA. To prepare FITC-BSA/PDA NPs, 80 mg of dehydrated foam of pristine BSA/PDA NPs were dissolved into 8 mL of sodium carbonate buffer (50 mM, pH 8.5). A concentrated solution of FITC (8.25 mM) was prepared by dissolving 3.2 mg into 1 mL of DMSO. 400 µL of this solution was added to the pristine BSA/PDA NPs solution to reach a FITC concentration of $4 \times 10^{-4}$ M ($3.3 \times 10^{-6}$ mol, 3 equiv). The reaction was performed in the dark for 1 h and followed by a dialysis against 1 L of Milli-Q® water using a Spectra/Por® dialysis membrane with a molecular weight cut-off at 8–10 kDa to eliminate unbound labelling molecules. The dialysis was done in the dark and repeated three times yielding a total dilution of the dialysate by a factor of $10^6$. The evolution of the dialysis was followed by measuring the absorption spectrum of the dialysate. The same procedure was used to prepare RhBITC-BSA/PDA NPs, except that FITC was replaced by RhBITC. The concentrated solution of RhBITC (8.25 mM) was prepared by dissolving 4.4 mg in 1 mL of DMSO.
NPs and RhBITC for Ox-BSA/PDA NPs and RhBITC-BSA/PDA NPs. Solutions of free FITC and RhBITC were prepared in water from the FITC (3.2 mg/mL) and RhBITC (4.4 mg/mL) solutions in DMSO to provide concentrations of 164 and 373 µM, respectively. For the characterization of Ox-PDA/BSA-NPs, 5 µL of 120 µM RhBITC solution was added to 95 µL of 3 mg/mL Ox-PDA/BSA-NPs solution in Tris according to Ma and co-workers [21]. The curves were smoothed by the centered moving average method on three points. Finally, the fluorescence quantum yield of Ox-BSA/PDA NPs was determined by comparison with a DAPI reference (4 µM) according to the relative method [33]. For the measurements, Ox-BSA/PDA NPs and DAPI solutions were both made using Milli-Q® water.

**Bacteriology**

**Bacterial species, strains, media, and culture conditions**

Gram-negative *Escherichia coli* (*E. coli*) and Gram-positive *Staphylococcus aureus* (*S. aureus*) species were used for antibacterial testing: *E. coli* K-12 PHL628 (purchased from Prof. Philippe Lejeune, INSA-Lyon, France) [43] and *S. aureus* ATCC 25923 (purchased from Pasteur Institute, France). Lysogeny broth (LB) (Merck, Darmstadt, Germany) (pH 6.8) and Mueller–Hinton broth (MH) (Merck, Darmstadt, Germany) (pH 7.4) were prepared in distilled water and were sterilized by autoclaving at 121 °C for 30 min before use. Bacteria were thawed, diluted, plated on agar plates (LB for *E. coli* and MH for *S. aureus*), and incubated under aerobic conditions for 24 h at 37 °C.

**Evaluation of the antibacterial activity**

The antibacterial activity of pristine NPs, Ox-, and RhBITC-BSA/PDA NPs with a BSA/DA ratio of 10 was determined by calculating the bacterial growth inhibition from absorption measurements at 620 nm. Measurements were acquired with a Multiskan spectrophotometer (Fisher Scientific, Illikich, France). One colony of *E. coli* or *S. aureus* was transferred from the agar plate into fresh liquid LB (for *E. coli*) or MH (for *S. aureus*) and incubated at 37 °C overnight. The bacterial suspension was then diluted with fresh LB (for *E. coli*) or MH (for *S. aureus*) to 6 × 10^6 CFU/mL. Lyophilized foam of BSA/PDA NPs with a BSA/DA ratio of 10 was diluted with 9 mg/mL NaCl solution to obtain a 20 mg/mL suspension of BSA/PDA NPs. This suspension was filtered in a microbiological safety cabinet with a 0.2 µm filter to eliminate possible contaminations and diluted in 9 mg/mL NaCl solution to prepare suspensions with NP concentrations of 0.5, 1, 2, 5, and 10 mg/mL. Finally, 10 µL of BSA/PDA NP suspension was added to 90 µL of bacterial solution (either *E. coli* or *S. aureus*) placed in 96 well plates to prepare bacterial suspensions with concentrations of BSA/PDA NPs from 0 to 2000 µg/mL (0 µg/mL (negative control C−), 50 µg/mL, 100 µg/mL, 200 µg/mL, 500 µg/mL, 1000 µg/mL, and 2000 µg/mL). A 10 µL mixture of tetracycline (10 µg/mL) and cefotaxime (0.1 µg/mL) was used as a positive control (C+) of bacterial growth inhibition. Bacterial growth was assessed from the absorption at 620 nm after 24 h of incubation at 37 °C. Each assay was performed three times. The statistical significance of two-by-two differences between the mean absorption at 620 nm was determined by unilateral Student’s tests. Bacterial growth inhibition was calculated for each concentration of NPs via Equation 4.

\[
\text{Inhibition (\%)} = \left(1 - \frac{A_{\text{bact+NPs}} - A_{\text{NPs}}}{A_{\text{bact}} - A_{\text{LB}}} \right) \times 100, \tag{4}
\]

where \(A_{\text{bact+NPs}}, A_{\text{NPs}}, A_{\text{bact}},\) and \(A_{\text{LB}}\) are the absorption values at 620 nm of the bacterial suspension containing NPs at a given concentration \((A_{\text{bact+NPs}})\), the bacteria-free suspension containing NPs at a given concentration \((A_{\text{NPs}})\), the bacterial suspension without NPs \((A_{\text{bact}})\), and the suspension with neither bacteria nor NPs \((A_{\text{LB}})\), respectively.

The minimal inhibitory concentration (MIC) corresponds to the lowest concentration of NPs that significantly inhibits growth of bacteria. If no inhibition effect is observed in the range of concentration tested, then the MIC will be indicated as higher than the maximum concentration tested.

**Determination of the localization of NPs in bacterial cells**

Standard and high-resolution fluorescence confocal laser scanning microscopy (CLSM) were used to determine the localization of the fluorescent BSA/PDA NPs with a BSA/DA ratio of 10 in bacterial cells. One colony of *E. coli* was transferred from the agar plate into fresh liquid LB and incubated at 37 °C overnight. The bacterial suspension was then diluted with LB to obtain a transmittance at 600 nm of 0.001 \((6 \times 10^6 \text{ CFU/mL})\). 200 µL of fluorescent Ox−, FITC−, or RhBITC-BSA/PDA NPs suspension was added to 800 µL of the bacterial suspension containing NPs at a given concentration \((A_{\text{bact+NPs}})\), the bacteria-free suspension containing NPs at a given concentration \((A_{\text{NPs}})\), the bacterial suspension without NPs \((A_{\text{bact}})\), and the suspension with neither bacteria nor NPs \((A_{\text{LB}})\), respectively. Controls were also prepared: one with bacteria alone, one with NPs alone, and one with bacteria, without NPs but with fluorescent dye, either FITC or RhBITC, in a concentration equivalent to the quantity used to label the NPs. Because PDA absorbed a significant part of the light emitted by the fluorophores, the emission could only be roughly estimated by the difference between the intensity of the dye solution added to label BSA/PDA NPs and the intensity of the dialysis water. The equivalent dye concentrations (corre-
Table 2: Samples prepared for fluorescence confocal microscopy.

<table>
<thead>
<tr>
<th>Sample composition</th>
<th>E. coli concentration (CFU/mL)</th>
<th>NP concentration (mg/mL)</th>
<th>Fluorescent dye concentration (in NPs or free) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA/PDA NPs + E. coli</td>
<td>6 × 10⁶</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Ox- BSA/PDA NPs + E. coli</td>
<td>6 × 10⁶</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>FITC-BSA/PDA NPs + E. coli</td>
<td>6 × 10⁶</td>
<td>2</td>
<td>164ᵃ</td>
</tr>
<tr>
<td>RhBITC-BSA/PDA NPs + E. coli</td>
<td>6 × 10⁶</td>
<td>2</td>
<td>373ᵃ</td>
</tr>
<tr>
<td>BSA/PDA NPs</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Ox-BSA/PDA NPs</td>
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<td>0</td>
</tr>
<tr>
<td>FITC- BSA/PDA NPs</td>
<td>0</td>
<td>2</td>
<td>164ᵃ</td>
</tr>
<tr>
<td>RhBITC-BSA/PDA NPs</td>
<td>0</td>
<td>2</td>
<td>373ᵃ</td>
</tr>
<tr>
<td>FITC + E. coli</td>
<td>6 × 10⁶</td>
<td>0</td>
<td>164</td>
</tr>
<tr>
<td>RhBITC + E. coli</td>
<td>6 × 10⁶</td>
<td>0</td>
<td>373</td>
</tr>
<tr>
<td>E. coli</td>
<td>6 × 10⁶</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

ᵃEstimation based on the difference in absorption intensity of the fluorescent dye solution used to label BSA/PDA NPs and the absorption intensity of the dialysis water.
42. Rang, C. U.; Peng, A. Y.; Poon, A. F.; Chao, L.
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Berberine-loaded polylactic acid nanofiber scaffold as a drug delivery system: The relationship between chemical characteristics, drug-release behavior, and antibacterial efficiency

Le Thi Le¹, Hue Thi Nguyen¹, Liem Thanh Nguyen², Huy Quang Tran¹,³ and Thuy Thi Thu Nguyen*¹,§

Abstract

Hydrophobic berberine powder (BBR) and hydrophilic BBR nanoparticles (BBR NPs) were loaded into an electrospun polylactic acid (PLA) nanofiber scaffold for modulating the release behavior of BBR in an aqueous medium. The BBR release from the BBR/PLA and BBR NPs/PLA nanofiber scaffolds was investigated in relation to their chemical characteristics, BBR dispersion into nanofibers, and wettability. The BBR release profiles strongly influenced the antibacterial efficiency of the scaffolds over time. When the BBR was loaded, the BBR/PLA nanofiber scaffold exhibited an extremely hydrophobic feature, causing a triphasic release profile in which only 9.8 wt % of the loaded BBR was released in the first 24 h. This resulted in a negligible inhibitory effect against methicillin-resistant Staphylococcus aureus bacteria. Meanwhile, the BBR NPs/PLA nanofiber scaffold had more wettability and higher concentration of BBR NPs dispersed on the surface of PLA nanofibers. This led to a sustained release of 75 wt % of the loaded BBR during the first 24 h, and consequently boosted the antibacterial effectiveness. Moreover, the cytotoxicity test revealed that the BBR NPs/PLA nanofiber scaffold did not induce any changes in morphology and proliferation of MA-104 cell monolayers. It suggests that the BBR/PLA and BBR NPs/PLA nanofiber scaffolds can be used in different biomedical applications, such as wound dressing, drug delivery systems, and tissue engineering, according to the requirement of BBR concentration for the desired therapeutic effects.
Introduction

Medicinal plants have various biologically active compounds, such as phenolic acids, alkaloids, saponins, coumarins, flavonoids, terpenoids, and carotenoids with great therapeutic effects [1]. Berberine (BBR) is a quaternary isoquinoline alkaloid, extracted from different medicinal plants, such as *Coptis chinensis* and *Berberis vulgaris* [2], and used in the treatment of central nervous system disorders [2], digestive system diseases [3], cancer, diabetes, inflammation, and infections. Nevertheless, BBR has a low bioavailability due to its poor water solubility, which imposes a regular intake of BBR drugs at a high dose. Recently, innovative technologies have been employed to produce nanoformulations of drugs for endowing a better therapeutic effect. The nanoformulations for drug delivery can be designed using nanocarrier systems, including organic materials (liposomes, nanoeumulsions, nanomicelles, and nanofibers) and inorganic nanoparticles (gold, silver, iron oxide, and mesoporous silica nanoparticles) [4]. Additionally, nanocarrier-free systems, such as drug nanocrystals, are also used to improve the delivery of poorly soluble drugs [5,6]. In our previous study, the saturation concentration of BBR in water was 2.0 mg/mL, while BBR nanoparticles prepared by antisolvent precipitation could reach up to 5.0 mg/mL, which notably increased the antibacterial activity of BBR [7].

Electrospinning is a convenient technique that allows one to fabricate nanofiber scaffolds with various compositions and structures. During the electrospinning process, a polymer solution blended with additional components is applied under a high-voltage electrostatic field, generating a charged and stretched solution jet following nanofiber formation [8,9]. Drug delivery systems based on nanofiber scaffolds produced by electrospinning method have strongly attracted researchers due to their unique characteristics. First, high porosity and large surface-to-volume ratio of nanofiber scaffolds give the material the potential to be exposed to the biological media for drug release. Besides, 3D nanofiber scaffolds resemble the natural extracellular matrix, promoting nutrients and cells to penetrate into their structure [10]. Second, high drug loading can be achieved, and the drug-release profile (i.e., prolonged, stimulus-activated, and biphasic releases) can be modulated by using different nanofiber structures (e.g., blending, core/shell, and multilayer structures) and nanofiber compositions [11-13]. For a long-term drug release, hydrophobic polymers are chosen for the preparation of drug-loaded nanofiber scaffolds. This is because the hydrophobicity of the polymer could form air gaps, slowing matrix hydration and suppressing drug diffusion from the nanofibers [14]. The core/shell nanofiber structure can also prolong the drug release since the polymer shell plays a role as a rate-control barrier [15]. On the other hand, the nanofiber scaffolds fabricated using suitable hydrophilic or water-soluble polymers are used to improve the dissolution profile and bioavailability of poorly soluble drugs [15]. Limoe et al. reported that the release rate of Pramipexole from hybrid cross-linked nanofibers was successfully controlled between 8 and 10 h, which is approximately the same time that the drug formulation travels from the mouth to the small intestine. It is worthwhile mentioning that the hybrid cross-linked nanofibers in this work were made of a mixture of a hydrophilic polymeric matrix (polyvinyl alcohol and carboxymethyl cellulose) and a hydrophobic polymer (polycaprolactone) [16]. Interestingly, the nature of the drug and the drug–polymer compatibility strongly affect the release behavior of the drug from nanofiber scaffolds [14,17-19]. Polyactic acid (PLA), a synthetic polymer that has been approved by the FDA for biomedical usage, is commonly used for drug delivery systems due to its biocompatibility and biodegradability [20]. Yuan et al. [17] explained the difference in the drug-release profile of PLA nanofibers loaded with hydrophobic doxorubicin hydrochloride (Dox-HCl) and hydrophobic free base doxorubicin (Dox-base). The rapid release of Dox-HCl from the PLA nanofiber carrier was attributed to Dox-HCl crystal aggregates mainly distributed on the surface of the fibers. In addition, the reduction in the hydrophobicity of the nanofiber network also caused a faster release of the drug. Meanwhile, the hydrophobic PLA nanofiber carrier showed a sustained release behavior of the Dox-base. This was because hydrophobic Dox-base significantly improved the miscibility with the PLA, forming a uniform drug dispersion in the PLA matrix and, therefore, restricting the drug release.

One of the main issues in clinical treatments is bacterial infections, which prolong treatment time or cause further complications. Among various types of nanomaterials, nanofiber scaffolds can act as a multifunctional tool in medical treatments, combining drug release for disease therapy, cell proliferation, wound healing, and antimicrobial effect [21-25]. Nanofibers of PLA functionalized with laponite (LAP)/amoxicillin (AMX) prolonged the drug release up to 21 days and inhibited the growth of *Staphylococcus aureus* and *Escherichia coli* bacteria. Human bone marrow mesenchymal stem cells were well attached and proliferated on the surface of the LAP/AMX functionalized PLA scaffolds, which provided a bacteria-free environment for bone differentiation in the treatment of bone defects [21]. In dentistry, anti-infective nanofiber-based drug-release systems have been investigated for periodontal disease control, endodontic therapy, cariogenic microorganism control, and tissue reconstruction [25]. Due to the controlled drug release, BBR-loaded nanofiber scaffolds exhibited excellent performance in repairing bone defects [3,26], healing diabetic foot ulcers [27], promoting hemostasis [28], acting as anti-leishmanial drugs [29], and inhibiting microbial agents [27,30].
Zhou et al. [31] developed hybrids of nanofibers and microparticles for dual-step controlled release of BBR, combining a fast-release step of BBR from hydrophilic polypyrrolidone nanofibers (47.9 wt % in the first hour) and a sustained-release step of BBR from the insoluble cellulose acetate microparticles (98.6 wt % for 60 h). In comparison with the aforementioned hybrid nanofibers, the release rate of BBR from PCL nanofibers [28] was significantly lower with an initial BBR release of 38 wt % in the first day, and a subsequent sustained BBR release of 76% during seven days. Meanwhile, a lower burst release of BBR from PCL/collagen nanofibers [3] was achieved on the first day (14.83 wt %) and this scaffold could prolong the release of BBR up to 27 days (81.4 wt %). The difference in BBR release profiles of these nanofiber scaffolds can be attributed to the difference in chemical characteristics of the polymer matrix, the content of BBR in the nanofibers, and the morphology of the nanofibers.

The release profile of antimicrobial agents should be adjusted depending on the infection conditions, such that a burst release mode is required for acute microbial infections, while slow and long-term release is more adequate to treat chronic infections [32]. This study aims to investigate the drug-release behavior of BBR from the electrospun PLA nanofiber scaffold, regarding drug–polymer compatibility and hydrophobicity of the scaffold. Besides, the antibacterial activity of these scaffolds relating to the release of BBR during 24 h was examined against methicillin-resistant Staphylococcus aureus (MRSA). The PLA nanofiber scaffold loaded with hydrophilic BBR nanoparticles showed faster BBR release compared with that of the hydrophobic BBR powder-loaded scaffold, resulting in better inhibitory effects against MRSA. The findings of this study suggest controlled drug-release profiles from nanofiber-based drug delivery systems for specific applications.

Result and Discussion
Morphology of PLA and BBR-loaded PLA nanofiber scaffolds
In order to evaluate the distribution of BBR compositions in the electrospun PLA nanofibers, the morphology of BBR powder, BBR NPs, and electrospun nanofibers was observed by scanning electron microscopy (SEM, Figure 1). The BBR powder appeared as aggregates of rods in the micrometer size (Figure 1a), while BBR NPs were formed as nanoscale rectangles (Figure 1b). The electrospun PLA nanofibers showed bead-
free and uniform morphology with fiber diameter in the range of 200–600 nm. The addition of BBR powder did not strongly affect the morphology of electrospun BBR/PLA nanofibers, except for a slight decrease in the average fiber diameter as shown in Table 1. This indicates that the hydrophobic BBR powder was well dissolved in the hydrophobic PLA polymer in the mixture of dichloromethane and N,N-dimethylformamide (DCM/DMF) solvent. Meanwhile, electrospun PLA nanofibers incorporated with BBR NPs had a wider diameter distribution with the addition of fiber diameters below 200 nm. Additionally, the BBR NPs/PLA nanofibers were more entangled and less uniform compared with BBR/PLA nanofibers. There is a possibility that BBR NPs were more hydrophilic than the BBR powder, leading to lower compatibility in the hydrophobic PLA polymer [17,33]. The incorporation of the BBR drug in PLA nanofibers resulted in a smaller fiber diameter, which was attributed to positively charged quaternary ammonium groups of BBR, increasing the charge density of the blend solution. As the higher charged solution jet, the elongation force imposed on the jet was higher, forming smaller fibers [3,34]. Interestingly, although the same amount of BBR drug was incorporated in the BBR/PLA and BBR NPs/PLA electrospun nanofiber scaffolds, the latter appeared with a darker yellow color, which is typical of the natural color of BBR (Figure 1c).

### Chemical characteristics and wettability of BBR-loaded PLA nanofiber scaffolds

By identifying distinct vibrational modes of various chemical bonds, Fourier transform infrared spectroscopy (FTIR) was used to examine the differences in chemical characteristics of BBR-loaded PLA nanofiber scaffolds (Figure 2A). The FTIR spectrum of PLA nanofiber scaffold shows the adsorption peaks at 1751 cm$^{-1}$ resulting from the stretching vibrations of the C=O bond in carboxylic groups. The two bands at 1182 cm$^{-1}$ and 1087 cm$^{-1}$ were attributed to the C–O–C binding vibrations. The absorption bands at 2992 cm$^{-1}$ and 2947 cm$^{-1}$ were characteristics of asymmetrical and symmetrical stretching vibrations of the C–H bond, while the asymmetrical vibrations of –CH$_3$ appeared at 1453 and 1358 cm$^{-1}$. These aforementioned adsorption characteristics of PLA nanofiber scaffolds were also observed in the FTIR spectra of BBR/PLA and BBR NPs/PLA nanofiber scaffolds. However, there was the addition of an intense broadband at 3361 cm$^{-1}$ in the FTIR spectrum of the BBR NPs/PLA nanofiber scaffold. This was due to the O–H

**Table 1:** Average diameter and water contact angle of electrospun PLA, BBR/PLA, and BBR NPs/PLA nanofiber scaffolds.

<table>
<thead>
<tr>
<th>Type of nanofiber scaffold</th>
<th>Average diameter (nm)</th>
<th>Water contact angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA</td>
<td>395 ± 78</td>
<td>130.1 ± 1.3</td>
</tr>
<tr>
<td>BBR/PLA</td>
<td>351 ± 65</td>
<td>126.3 ± 1.6</td>
</tr>
<tr>
<td>BBR NPs/PLA</td>
<td>356 ± 98</td>
<td>107.0 ± 2.2</td>
</tr>
</tbody>
</table>

**Figure 2:** FTIR spectra of (a) PLA, (b) BBR/PLA, and (c) BBR NPs/PLA nanofiber scaffolds at different wavenumber ranges (A, B, C).
stretching vibration of the glycerol component in BBR NPs (Figure 2). Peculiarly, the absorption bands at 1646 cm$^{-1}$ and 1506 cm$^{-1}$, characteristic of the C=N$^+$ double bond and the furyl group in the molecular structure of BBR, respectively, were only displayed in the FTIR spectrum of the BBR NPs/PLA nanofiber scaffold. This is evidence that the chemical characteristics of BBR on the BBR NPs/PLA nanofibers were detected more clearly than that of the BBR/PLA nanofibers at the same amount of BBR incorporated into nanofibers, possibly due to the higher concentration of the BBR molecule on the surface of BBR NPs/PLA nanofibers. The observation of Figure 1c further supports this assumption.

The analysis of Raman spectra (Figure 3A) was employed to confirm chemical characteristics of PLA, BBR/PLA, and BBR NPs/PLA nanofiber scaffolds. The distinct peaks of the PLA nanofiber scaffold were found at 887, 1046, 1129, 1305, 1458, 1766, and 2948 cm$^{-1}$ corresponding to the vibration of $\nu$C–COO stretching, $\nu$C$\alpha$–C$\beta$ stretching, $\delta$CH$_3$ rocking, $\delta$CH bending, $\delta$CH$_3$ asymmetric deformation, $\nu$C=O stretching, and $\nu$CH$_3$ stretching modes [35,36]. In the case of BBR drug-loaded PLA nanofiber scaffolds, there were peaks at 234, 531, 1388, and 1636 cm$^{-1}$ characteristic of BBR [7,37]. However, the intensity of the characteristic peaks of BBR loaded in PLA nanofiber scaffold was markedly decreased compared with those of BBR NPs loaded in the PLA nanofiber scaffold. This evidence strongly supports the conclusion that the BBR concentration on the surface of BBR NPs/PLA nanofibers was higher than that on the surface of BBR/PLA nanofibers, which is in agreement with the above FTIR analysis. The poor miscibility between the hydrophilic drug and the hydrophobic polymer might cause phase separation during the electrospinning process [17,38,39], leading to the formation of a BBR-rich phase on the surface of nanofibers.

The crystallinity of the PLA pellet and electrosupn nanofiber scaffolds were examined by X-ray diffraction (XRD) analysis (Figure 3B). The XRD pattern of the PLA pellet shows diffraction peaks at 20 of 16.7, 19.2, and 22.4° associated to a crystalline $\alpha$-form orthorhombic structure (card number 00-0541917, Diffract Plus 2005), while the weak diffraction peaks at 28.9 and 30.9° were characteristic of the $\beta$-form trigonal structure [40]. The XRD pattern of the PLA nanofibers formed after stretching the PLA solution during the electrospinning process
displays only a broad scattering band, located at around $2\theta = 21.3^\circ$, indicating an amorphous structure of PLA nanofibers. Due to the stretching and rapid solidification of the PLA solution during traveling from the needle to the collector, the rearrangement of the polymer chains into lamellar packing was limited, resulting in domination of the amorphous region in PLA nanofibers [41]. The XRD patterns of BBR drug-loaded PLA nanofiber scaffolds exhibit a distinct peak similar to that of the PLA nanofiber scaffold without the appearance of the characteristic peaks of BBR NPs at 6.79°, 9.13°, and 13.90° as reported in a previous study [42].

The wettability of the drug-loaded nanofiber scaffolds is an important factor affecting their drug-release behavior. It is reported that the high hydrophobicity of drug-loaded nanofibers resulted in prolonged drug release due to the delayed penetration of water into the polymer scaffolds. The change in the water contact angle of PLA nanofiber scaffolds loaded with BBR powder and BBR NPs is presented in Table 1. The PLA nanofiber scaffold possessed typical hydrophobic property with a water contact angle value of 130.1 ± 1.3°. This value was slightly decreased to 126.3 ± 1.6° when the BBR powder was added to the nanofibers. Meanwhile, the water contact angle value of the BBR NPs/PLA nanofiber scaffold was reduced by 23° relative to that of the PLA nanofiber scaffold, attributing to the hydrophilic BBR NPs favorably concentrated on the surface of the nanofibers. The relationship between the wettability and the BBR-release behavior of BBR-loaded PLA nanofiber scaffolds will be reported in the following drug-release profiles.

**In vitro drug-release profiles and release kinetics**

In vitro release profiles of BBR from BBR/PLA and BBR NPs/PLA nanofiber scaffolds were showed in Figure 4. It can be seen that BBR/PLA and BBR NPs/PLA nanofiber scaffolds exhibited different drug-release characteristics, which were triphasic and sustained BBR-release profiles, respectively. In the case of the BBR/PLA nanofiber scaffold, a slow release of BBR was observed during the first 24 h (lag time), attributed to the hydrophobicity of the scaffold requiring a long time for water permeation. When the scaffold was wetted, BBR was fast released, reaching approximately 60% of the loaded BBR in 36 h. However, in the next 28 h, the BBR/PLA nanofiber scaffold additionally released 15% of the BBR loaded at a slow rate, possibly due to the hardly diffused out BBR embedded in the core region of the nanofibers. As discussed above, the wettability of the BBR NPs/PLA nanofiber scaffold was significantly enhanced. Hence, the BBR was gradually released from the BBR NPs/PLA nanofiber scaffold within 64 h without lag time, and the final release percentage reached a high value of 93%. It is worth mentioning that the release profiles of BBR from BBR/PLA and BBR NPs/PLA nanofiber scaffolds may be suitable for various applications which require different release behaviors for desired therapeutic effects. Ma et al. [3] reported that...
the prolonged release of BBR from PCL/collagen nanofiber scaffolds up to 27 days was favorable for bone tissue repair. Meanwhile, a high concentration of BBR release within the first 24 h brought good antibacterial activity for wound dressing [28,30].

In order to study the mechanism of BBR release from BBR/PLA and BBR NPs/PLA nanofiber scaffolds, the release data were fitted to several kinetic models, including zero-order, first-order, Higuchi, and Ritger–Peppas models. The regression equations and parameters determined by fitting the BBR release data to the aforementioned mathematical models are shown in Supporting Information File 1 and Table 2. The correlation coefficient ($R^2$) of BBR release from the BBR/PLA nanofiber scaffold was the largest when fitted to the Ritger–Peppas model compared to that of other models. In addition, the release exponent ($n$) of the equation was 0.1703, indicating that the BBR release from the BBR/PLA nanofiber scaffold followed the Fickian diffusion. In this mechanism, the release of BBR was governed by a diffusion process, where the diffusion rate was higher than the polymer relaxation [43]. Based on the $R^2$ values shown in Table 1, the release data of the BBR NPs/PLA nanofiber scaffold was simultaneously well described by the Higuchi and Ritger–Peppas models, suggesting that BBR NPs release was mainly controlled by a diffusion mechanism. However, the value of $n$ determined by the Ritger–Peppas model was in the range of 0.5 and 1.0, which means that the BBR NPs release mechanism could be represented by a non-Fickian diffusion. In other words, the release of BBR NPs was not only based on diffusion but also involved other processes, such as dissolution or degradation of BBR NPs largely concentrated on the surface of the BBR NPs/PLA nanofiber scaffold [43]. In conclusion, the physiochemical properties of the BBR drug greatly affected the distribution of BBR on the PLA nanofibers, subsequently resulting in different BBR release profiles and mechanisms.

The mechanism of BBR release from BBR/PLA and BBR NPs/PLA nanofiber scaffolds was proposed in Figure 5. Since the degradation of PLA nanofibers is a long-term process (over a week) in buffer solution [17], the release of BBR from PLA nanofiber scaffold might be mainly dominated by the distribution of BBR in the nanofibers and the fiber wettability. In the case of hydrophobic BBR dispersed in hydrophobic PLA nanofibers, the release of BBR could take place in three main steps: (1) water molecules diffused from the aqueous medium onto the surface of nanofibers, dissolved the BBR molecules embedded on the surface of nanofibers, and then the dissolved BBR molecules were diffused into the medium. This step took a long time due to the high hydrophobicity of BBR/PLA nanofibers, causing a lag time in the initial stage of release. (2) When the PLA nanofibers were wetted, water molecules penetrated into the nanofibers, resulting in a high release of BBR by diffusion. (3) The BBR molecules in the core of PLA nanofibers slowly diffused out over a prolonged time. Meanwhile, the mechanism of BBR release from BBR NPs/PLA nanofiber scaffolds occurred in two main steps: (1') water molecules from the aqueous medium quickly diffused and dissolved the BBR molecules embedded on the surface of nanofibers due to their higher hydrophilicity. (2') The BBR molecules were continuously released thanks to the high concentration of BBR located near the surface of PLA nanofibers. As a result, pores could be formed in the nanofiber matrix, which could allow the gradual diffusion of BBR inside the nanofibers to the aqueous medium.

**Antibacterial performance of BBR-loaded nanofiber scaffolds**

To evaluate the antibacterial efficiency of BBR-loaded nanofiber scaffolds in the relationship with their BBR release profiles, the antibacterial test of these scaffolds against MRSA was performed during 24 h. The antibacterial activity was accessed by the bacterial concentration in the incubation solutions at each time interval corresponding to the amount of released BBR. Figure 6 and Supporting Information File 2 present the negative control and the antibacterial effectiveness of BBR/PLA and BBR NPs/PLA nanofiber scaffolds against MRSA at each time interval. The growth curve of MRSA incubated in the nutrient broth during 24 h shows two distinct phases of bacterial growth, which are exponential and stationary phases. The exponential phase occurred in the first 12 h when the cell numbers were doubled after each generation time. After that,

### Table 2: Parameters determined by fitting BBR release data to four different mathematical models.

<table>
<thead>
<tr>
<th>Mathematical model</th>
<th>Zero order</th>
<th>First order</th>
<th>Higuchi</th>
<th>Ritger–Peppas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_0 = 0.0145$</td>
<td>$K_1 = 0.0836$</td>
<td>$K_4 = 14.69$</td>
<td>$K_R = 2.1457$</td>
</tr>
<tr>
<td>$R^2 = 0.9071$</td>
<td>$R^2 = 0.7516$</td>
<td>$R^2 = 0.8959$</td>
<td>$n = 0.1703$</td>
<td>$n = 0.9374$</td>
</tr>
<tr>
<td>BBR/PLA nanofiber scaffold</td>
<td>$K_0 = 0.0099$</td>
<td>$K_1 = 0.0164$</td>
<td>$K_4 = 10.623$</td>
<td>$K_R = 0.4278$</td>
</tr>
<tr>
<td>$R^2 = 0.8385$</td>
<td>$R^2 = 0.7075$</td>
<td>$R^2 = 0.9295$</td>
<td>$n = 0.8616$</td>
<td>$R^2 = 0.918$</td>
</tr>
</tbody>
</table>

the stationary phase was reached when the number of growth cells was almost equal to that of dead cells. The proliferation of MRSA incubated in the nutrient broth with the presence of the BBR/PLA nanofiber scaffold also exhibited these two phases. The weak inhibitory effect of the BBR/PLA nanofiber scaffold against MRSA was observed over the period of 3 and 9 h. This could be explained by the small amount of BBR released from the BBR/PLA nanofiber scaffold during the first 12 h, as mentioned in the drug-release results. Meanwhile, a notable decrease in MRSA growth for 24 h was clearly achieved when MRSA was treated with the BBR NPs/PLA nanofiber scaffold. In addition, the number of MRSA cells was not significantly different between 12 and 24 h, indicating that the stationary phase was reached faster due to the inhibitory activity of the BBR NPs/PLA nanofiber scaffold. It is a fact that the BBR NPs/PLA nanofiber scaffold could sustainably release a high amount of BBR (75%) during 24 h, resulting in the boosted antibacterial effectiveness of the scaffold. A previous study reported that BBR exhibited excellent antibacterial activity against MRSA by damaging the cell wall structure and membrane integrity and further changing the cell morphology in the concentration range of 64–256 mg/L [44]. Recently, Wu et al. [45] proposed a novel orientation on the antibacterial mechanism of BBR against a standard strain Staphylococcus aureus, whereby BBR inhibits the synthesis of the cell wall and an aromatic amino acid induces oxidative damage and decreases stress resistance. Besides, BBR was found to inhibit MRSA biofilm formation with the concentration in the range of 1–64 mg/L [46]. In our study, the concentration of BBR released from BBR NPs/PLA nanofiber scaffolds after 6, 12, and 24 h was 87.8, 106.0, and 150.5 mg/L, respectively, which are in the BBR concentration range, leading to an inhibitory effect against MRSA similarly to the aforementioned studies.

**Cytotoxicity of BBR NPs/PLA nanofiber scaffolds**

Microbiological associates-104 (MA-104) cell monolayers were cultured with the BBR NPs/PLA nanofiber scaffold, which was cut and fitted to the bottom of the wells of a plastic 96-well plate filled with DMEM. The cells were incubated for 120 h and photographed under a microscopy at each time interval. Figure 7 shows the microscopic examination of MA-104 cells incubated with and without the presence of BBR NPs/PLA nanofiber scaffold. In addition, the number of MRSA cells was not significantly different between 12 and 24 h, indicating that the stationary phase was reached faster due to the inhibitory activity of BBR NPs/PLA nanofiber scaffolds.
Figure 6: The growth curves of MRSA incubated in nutrient broth (negative control) and treated with BBR/PLA and BBR NPs/PLA nanofiber scaffolds. Different letters indicate significant differences ($p < 0.05$) between groups, whereas the same letter denotes that the differences between groups are nonsignificant ($p > 0.05$). Statistical results were obtained from one-way analysis of variance (ANOVA).

cells treated with the BBR NPs/PLA nanofiber scaffold (Figure 7b) had a similar cell morphology, suggesting that this scaffold did not exhibit cytotoxic activity against MA-104 cells. Therefore, it is proposed that the BBR NPs/PLA nanofiber scaffold can be a potential candidate for broad biomedical applications, such as wound dressing, drug delivery, and tissue engineering.

**Conclusion**

PLA nanofiber scaffolds loaded with BBR powder and BBR NPs were fabricated by electrospinning technique. The average diameter of BBR/PLA and BBR NPs/PLA nanofibers were 351 ± 65 and 356 ± 98 nm, respectively. The chemical characteristics, BBR dispersion into the nanofibers, and fiber wettability of these scaffolds depended on the compatibility of the BBR drug and PLA polymer. The poor compatibility of hydrophilic BBR NPs and hydrophobic PLA resulted in a higher concentration of BBR located on the surface of nanofibers and lower water contact angle value of the scaffold compared to that of the scaffold prepared by the blend of hydrophobic BBR powder and hydrophobic PLA. Consequently, the PLA nanofiber scaffold loaded with BBR NPs gradually released a maximum of 93% of BBR during 64 h and effectively inhibited the proliferation of MRSA during 24 h. Meanwhile, the BBR concentration released from the BBR/PLA nanofiber scaffold during the first 24 h did not reach the minimum inhibition concentration for MRSA. The release of BBR from PLA nanofiber scaffolds was best fit with Ritger–Peppas models, suggesting that BBR release was mainly controlled by a diffusion mechanism. Additionally, the BBR NPs/PLA nanofiber scaffold did not exhibit cytotoxic activity against MA-104 monolayer cells. Different BBR release profiles reported in this study can be suitable design for different applications requiring a certain range of therapeutic concentrations of BBR.

**Experimental Materials**

Polylactic acid pellets ($M_w$ of 50,000, purity > 98%) were purchased from Total Corbion (Netherlands). N,N-Dimethylformamide (≥ 99.5%) and dichloromethane (>98%) were supplied by Xilong Scientific Co., Ltd., China. Berberine chloride powder (purity > 99%, pharmaceutical primary standard) was commercially obtained from Sigma-Aldrich, Singapore. Nutrient broths were provided by Titan Biotech, India. Bi-distilled water was used to prepare all solutions. All the chemicals were used without any purification.

Methicillin-resistant *Staphylococcus aureus* bacterial strain were isolated from clinical samples of hospitalized patients and stored according to Clinical and Laboratory Standards Institute (CLSI) regulations. The microbiological associates-104 cell line is an epithelial cell from fetal kidney of an African green monkey. The bacterial strain and the MA-104 cell line were provided by the National Institute of Hygiene and Epidemiology, Vietnam.

**Preparation of electrospun PLA, BBR/PLA and BBR NPs/PLA nanofiber scaffolds**

Berberine nanoparticles were formed through the antisolvent precipitation process described in our previous report [7]. A 7.0 wt % PLA solution was prepared by dissolving the PLA pellets in a solvent mixture of DCM/DMF with a weight ratio of 80/20 under magnetic stirring for 1 h at 50 °C. After that, the BBR powder and BBR NPs were separately added into the PLA solutions and continuously stirred for 2 h at 40 °C to obtain yellow clear solutions of BBR/PLA and BBR NPs/PLA, respectively. The amount of BBR in these solutions was calculated as 1.0 wt % of PLA composition.

Nanofiber scaffolds of PLA, BBR/PLA, and BBR NPs/PLA were fabricated through the electrospinning of the aforementioned prepared solutions. After the solutions were cooled down to room temperature, they were transferred to a 5 mL syringe with a 22-gauge stainless-steel needle. The needle was linked to a high-voltage power supply (Nano NC, Korea) to generate a 15 kV voltage for the electrospinning process. By using a microinjection pump, the flow rate of the solution through the needle was maintained at 1.0 mL/h. The distance between the needle tip and the roller collector was fixed at 18 cm. The electrospinning process was conducted for 6 h to obtain the PLA, BBR/PLA, and BBR NPs/PLA nanofiber scaffolds.
Characterization of prepared scaffolds
The morphology of PLA and BBR-loaded PLA nanofiber scaffolds was observed by a scanning electron microscope (JSM-6510LV). Fiber diameters were measured from the SEM images by using the ImageJ software as an image analysis tool.

Fourier-transform infrared spectroscopy was performed in a Nicolet NEXUS 670 spectrometer. The resulting spectra were recorded in transmission mode in the wavelength range of 500–4000 cm\(^{-1}\).

A Raman spectrometer (MacroRAM, Horiba) was used to investigate the chemical characteristics of prepared scaffolds in the wavelength range of 200–3200 cm\(^{-1}\). X-ray diffraction measurements of PLA pellets and BBR-loaded PLA nanofiber scaffolds were analyzed with Cu K\(\alpha\) radiation in a 2\(\theta\) range from 5 to 80\(^\circ\) using EQUINOX 5000 – Thermo Scientific X-ray diffractometer.

Static contact angles of the electrospun scaffolds were measured using a Samsung FACED camera (Korea). A drop of bidistilled water was placed on the flat surface of the electrospun scaffold and then a digital image of the drop was taken for measuring the value of the contact angle using an image processing program. All samples were measured at least five times from different locations and the average value was reported.

In vitro drug-release study
The release of BBR from the scaffolds was performed in a nutrient solution, which was also used for the antibacterial testing in order to assess the relationship between their antibacterial activity with the BBR release profile from these scaffolds. To determine the concentration of BBR in the nutrient solution, a standard calibration curve of UV–vis absorbance versus BBR concentrations was built as follows: 1 mg of BBR powder was dissolved in 1 mL of bidistilled water to obtain a BBR solution stock. Then, this solution was diluted by the nutrient solution in volumetric flasks to make concentrations ranging from 1–200 µg/mL. The absorbance of these BBR solutions was read at 421 nm using a UV–vis spectrophotometer (6850 UV–vis, Jenway).

Electrospun PLA, BBR/PLA, and BBR NPs/PLA nanofiber scaffolds were employed for drug-release tests. The scaffolds were cut into a rectangular shape with a PLA weight of 0.1 g in all samples. Each sample was put in a 10 mL bottle containing 5 mL of nutrient solution. After that, the bottles were shaken using a PTR-35 Vertical Multi-function shaker at room temperature with constant agitation at 40 rpm. At each time interval, 2 mL of each solution was withdrawn, the UV–vis absorbance at 421 nm was measured, and then the amount of BBR release based on the standard calibration curve was calculated. The percentage of released BBR at each time interval was calculated by Equation 1. All the experiments were repeated three times.

\[
\text{Percentage of BBR release (\%) = } \frac{M_t}{M_m} \times 100\% \quad (1)
\]

In Equation 1, \(M_t\) (mg) is the weight of BBR released at each time interval and \(M_m\) (mg) is the weight of BBR incorporated in the scaffold.

Mathematical models
In order to distinguish the mechanism of BBR released from BBR/PLA and from BBR NPs/PLA nanofiber scaffolds, the data of the experimental BBR release were described by four kinetic models, including the zero-order model, first-order model, Higuchi model, and Ritger–Peppas model (Supporting Information File 1).

Antibacterial test
The antibacterial activity of BBR/PLA and BBR NPs/PLA nanofiber scaffolds was tested against MRSA (Gram-positive bacteria). The antibacterial tests were performed in a sterilized 20 mL glass tube containing bacterial solutions with a concentration of approx. 3 \(\times\) 10\(^3\) colony-forming units (CFU)/mL in nutrient broth. BBR/PLA and BBR NPs/PLA nanofiber scaffolds were cut into rectangles with a PLA weight of 0.1 g and then put into the test tubes. For a negative control, one tube was retained without any scaffold sample. Subsequently, all the test tubes were statically incubated at 37 °C for 24 h.

The proliferation of bacteria during 24 h of incubation with and without samples was evaluated by counting the bacterial colonies growing on the agar surface. Briefly, at each incubation time interval (0, 3, 6, 9, 12, 24 h), the solutions in each test tube were diluted many times in physiological saline. A volume of 100 µL of the initial and diluted solutions was spread onto the agar surface in plastic Petri discs and statically incubated at 37 °C for 24 h. Finally, the concentration of bacteria in inoculated solutions was calculated based on the number of bacterial colonies.

Test of cytotoxicity of BBR NPs/PLA nanofiber scaffolds
The cell culture medium was prepared by mixing 150 mL of Dulbecco’s modified eagle medium (DMEM) with 600 µL of 0.5 mg/mL trypsin in a Schott bottle. MA-104 cells were distributed into the wells of a 96-well plate in DMEM supplemented with 10% fetal bovine serum. The BBR NPs/PLA nanofiber scaffold was cut into circles with a diameter of 6 mm and sterilized with ultraviolet light for 12 h. Then these cut scaffolds...
were submerged in the cell culture medium in the 96-well plate and incubated for 120 h at 37 °C in an atmosphere of 5% CO₂. The morphology of the cultured cells was monitored with an inverted microscope every 24 h.

**Statistical analysis**

The data were reported as mean values ± standard deviations. Statistical analysis of antibacterial data was performed using one-way analysis of variance (ANOVA). A $p$-value of less than 0.05 was considered statistically significant.

**Supporting Information**

**Supporting Information File 1**

Mathematical models.  
[https://www.beilstein-journals.org/bjnano/content/supplementary/2190-4286-15-7-S1.pdf]

**Supporting Information File 2**

Photographs of agar plates inoculated with MRSA treated with BBR/PLA and BBR NPs/PLA nanofiber scaffolds and negative control.  
[https://www.beilstein-journals.org/bjnano/content/supplementary/2190-4286-15-7-S2.pdf]

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**Data Availability Statement**

All data that supports the findings of this study is available in the published article and/or the supporting information to this article.

**References**


Study of the reusability and stability of nylon nanofibres as an antibody immobilisation surface


Abstract

In the case of a biological threat, early, rapid, and specific detection is critical. In addition, ease of handling, use in the field, and low-cost production are important considerations. Immunological devices are able to respond to these needs. In the design of these immunological devices, surface antibody immobilisation is crucial. Nylon nanofibres have been described as a very good option because they allow for an increase in the surface-to-volume ratio, leading to an increase in immunocapture efficiency. In this paper, we want to deepen the study of other key points, such as the reuse and stability of these nanofibres, in order to assess their profitability. On the one hand, the reusability of nanofibres has been studied using different stripping treatments at different pH values on the nylon nanofibres with well-oriented antibodies anchored by protein A/G. Our study shows that stripping with glycine buffer pH 2.5 allows the nanofibres to be reused as long as protein A/G has been previously anchored, leaving both nanofibre and protein A/G unchanged. On the other hand, we investigated the stability of the nylon nanofibres. To achieve this, we analysed any loss of immunocapture ability of well-oriented antibodies anchored both to the nylon nanofibres and to a specialised surface with high protein binding capacity. The nanofibre immunocapture system maintained an unchanged immunocapture ability for a longer time than the specialised planar surface. In conclusion, nylon nanofibres seem to be a very good choice as an antibody immobilisation surface, offering not only higher immunocapture efficiency, but also more cost efficiency as they are reusable and stable.

Introduction

Biological threats involve a wide range of risks not only to the human population, but also to livestock and crops [1], affecting both human health (mortality, morbidity, and incapacity) and the economy (crop failures, livestock deaths, and investments in health and safety) [2]. For this reason, early, rapid, and specific detection of biological threats becomes a very important objec-
tive to react as early as possible. Many efforts have been made in this direction. When designing a new sensor device, not only the rapid and specific identification has to be taken into account, but also ease of handling, on-site use, and low production cost. Thus, several authors, such as Janik-Karpinska and colleagues in 2022 [1], have pointed out that rapid detection of pathogens and toxins in food, water, and the environment is a paramount health and safety need to reduce the risk of pandemic contamination. Early, reliable, and accurate diagnosis is therefore essential for health and food safety [4,5].

In this context, immunodetection seems to be a very good option [6]. There are many applications of immunoassay devices in health, food industry, and clinical applications. Immunoassay devices have been used not only for the detection of bacteria and viruses [7], but also for the measurement of drugs [8] and hormones [9], or for the determination of glucose in urine [10].

The specificity of antigen–antibody binding and how the antibody is attached to the biosensor surface, in terms of density, orientation, and stability, will determine the diagnosis capability of the device [11]. Thus, the immobilisation surface of the device is one of the key points in the development of new sensors.

Nylon has been used as immobilisation surface in numerous applications, such as the immobilisation of enzymes and microorganisms [12,13], and the immobilisation of antibody in enzyme immunoassays [14]. Nylon 6 (or polyamide 6, PA6) nanofibers (NFs) have been used as an immobilisation surface in biosensors [15]. Efficiency studies of nanofibres manufactured by electrospinning have been carried out in our laboratory, determining the optimal nanofibre thickness regarding stability and biofunctionalisation [16]. Our results showed that the NFs’ surface provides advantages over a planar nylon surface in terms of increased immunocapture efficiency as the higher surface area/volume ratio in the nanofibre allows for a greater amount of immobilised antibody in the same space [17]. In addition, some studies demonstrate the suitability of electrospun nylon NFs for the development of Fabry–Pérot-based optical biosensors [18,19]. However, for the selection of such NFs as immobilisation surfaces in biosensors, it seems necessary to study those characteristics of the immobilisation surface that contribute to their lower cost.

In this regard, this paper not only investigates the reuse of NFs, but also whether this immobilisation surface provides a longer life for an immunocapture system. These characteristics are key points to obtain a more cost-effective and environmentally friendly immobilisation surface.

One of the aims of developing a rapid and easy-to-use biosensor is to be able to detect a biological threat as early as possible. In the “Nanofibre reusability study” developed in this paper, bovine serum albumin (BSA) was chosen as a surrogate for biotoxins. In contrast, in the “Stability study” carried out in this paper, ricin was used as a representative biotoxin instead of a surrogate because the “Stability study” required less handling than the “Nanofibre reusability study”. Ricin has been chosen as a representative biotoxin because it has been used in biological warfare attacks because of its high toxicity, stability, and availability. It belongs to the ribosome-inactivating protein family and causes cell death by disrupting protein synthesis [20].

**Results and Discussion**

**Results of nanofibre reusability study**

High-salinity antigen/antibody (Ag/Ac) elution buffer pH 6.6 as stripping agent

A commercial Ag/Ac elution buffer pH 6.6 with high salinity was able to remove almost all antibody fixed on the nanofibres through protein A/G (88.6%). The retained antibody fraction after stripping treatment (group 2) was only 11.4% compared to the reference group 1, which is 100% (Figure 1).

The amount of bound antibody is indicated by the amount of fluorescein (FITC) fluorescence detected as this fluorochrome is associated with the antibody in question. It is measured as relative fluorescence unit (RFU) (index explained in the Experimental section). Results are expressed as the mean RFU of the replicates, and the variation is expressed as the standard error of the mean (SEM).

When the immunocapture system had been reconstituted after the stripping procedure (group 3), only 45.4% of bound antibody was found, compared to the total amount of antibody fixed in group 1 (Figure 1). This suggests that antibody binding was altered by the buffer. The same results were found when BSA alone (group 4) or antibody plus BSA (group 5) was administered after stripping treatment, yielding 28.1% and 31.9%, respectively, of the amount of antibody fixed in group 1 (Figure 1).

Bare NFs (group 6) are NFs that have undergone the stripping process without prior binding to the immunocapture system. These bare NFs were damaged to the extent that they were unable to bind the immunocapture system (15.5% compared to the total antibody fixed in group 1 (Figure 1). Hence, it seems that commercial Ag/Ac elution buffer pH 6.6 with high salt content damages the nylon nanofibers, thereby altering their immunocapture ability.
Having studied how the amount of immobilised antibody was affected, we also wanted to determine how the immunocapture capacity of these immobilised antibodies was affected. This was determined by assessing the fluorescence associated with the immunocaptured antigen, which, in this study, was the protein toxin simulant BSA. The BSA-associated fluorochrome was phycoerythrin (RPE), and the amount of immunocaptured antigen was assessed by the intensity of RPE fluorescence (measured in RFU).

Regarding the BSA immunocapture, the results showed some unspecific BSA binding after stripping treatment (group 3) as the amount bound antibody (45.4%) was less than that of immunocaptured BSA (60.9%), both values compared to group 1 (Figure 1 and Figure 2).

Similar results were obtained in the reconstituted immunocapture system when BSA alone (group 4) or antibody plus BSA (group 5) were administered after stripping. While 28.1% and 31.9% of bound antibody was found in groups 4 and 5, respectively, 40.9% and 65.5% of immunocaptured BSA was detected in these groups, both compared to group 1 (Figure 1 and Figure 2). Furthermore, after stripping treatment, the bare nanofibre (group 6) was only able to bind 15.5% of the total antibody initially bound, whereas 35.3% of the BSA was immunocaptured in this group (group 6) compared to group 1 (Figure 1 and Figure 2).

\[
\begin{array}{cccccc}
\text{Groups} & 1 & 2 & 3 & 4 & 5 & 6 \\
\text{Protein A/G} & + & + & + & + & + & - \\
\text{Antibody} & + & + & + & + & - & - \\
\text{BSA} & + & + & + & - & - & - \\
\text{Stripping with high salinity pH 6.6} & - & + & + & + & + & + \\
\end{array}
\]

\[
\begin{array}{cccccc}
\text{Groups} & 1 & 2 & 3 & 4 & 5 & 6 \\
\text{Protein A/G} & + & + & + & + & + & + \\
\text{Antibody} & - & - & + & - & - & + \\
\text{BSA} & - & - & - & + & + & + \\
\end{array}
\]

\[
\begin{array}{cccccc}
\text{Percentage from original system} & 100 & 11.4 & 45.4 & 28.1 & 31.9 & 15.5 \\
\text{Mean} & 18.4 & 5.1 & 13.9 & 7.4 & 3.7 & 4.1 \\
\text{SEM} & & & & & & \\
\end{array}
\]

Figure 1: FITC fluorescence of anti-BSA antibody. For each group, the FITC fluorescence data of the immobilised anti-BSA antibody, measured in RFU, are given as percentages relative to the reference group 1, \( n = 5–6 \). Stripping treatment with commercial Ag/AgCl elution buffer pH 6.6 was performed in all groups except group 1, which was used as the reference in the statistical analysis. One-way ANOVA followed by Newman–Keuls test. Difference from original immunocapture system fluorescence (reference group: group 1): *** \( p < 0.001 \).

Ammonium hydroxide buffer pH 11 as stripping agent

Ammonium hydroxide buffer pH 11 gave similar results to the commercial high-salinity Ag/AgCl elution buffer pH 6.6 (Figure 3).

This treatment was able to remove almost all of the antibody captured by the NFs via protein A/G (93.4%) since the retained
Figure 2: RPE fluorescence of immunocaptured BSA. For each group, the RPE fluorescence data of the immunocaptured BSA, measured in RFU, are given as percentages relative to the reference group 1, \( n = 5–6 \). Stripping treatment with commercial Ag/Ac elution buffer pH 6.6 was performed in all groups except group 1, which was used as the reference in the statistical analysis. One-way ANOVA followed by Newman–Keuls test. Difference from original immunocapture system fluorescence (group 1): **\( p < 0.01 \), ***\( p < 0.001 \).

Glycine buffer pH 2.5 as stripping agent

In contrast, buffer containing glycine pH 2.5 was able to remove 70% of the total fixed antibody since the retained anti-

antibody after stripping treatment (group 2) was only 6.6% compared to group 1 (Figure 3). However, the ammonium hydroxide buffer pH 11 interfered with the reconstituted immunocapture system to such an extent that only 31.5% of the captured antibody was detected after the reconstruction process (group 3) compared to group 1 (100%). When bare NFs were treated with the ammonium buffer (group 6), almost no antibody was bound (11.6%) (Figure 3).

As with the commercial high-salinity Ag/Ac elution buffer pH 6.6, non-specific binding of BSA was observed in NFs after stripping treatment (group 3) as more immunocaptured BSA was detected than bound antibody (Figure 4).

In group 3, while 31.5% of bound antibody was detected, 50.3% of BSA was immunocaptured, both values compared to group 1 (Figure 3 and Figure 4). After stripping, the reconstituted immunocapture systems after reapplying BSA only (group 4) or antibody plus BSA (group 5) exhibited 4.9% and 21.6% of bound antibody and 21.6% and 53.1% of immunocaptured BSA, respectively, compared to group 1 (Figure 3 and Figure 4). Furthermore, after buffer treatment, the bare NFs (group 6) were able to bind only 11.6% of the total antibody and immunocaptured 36% of BSA (Figure 3 and Figure 4).

Thus, both ammonium hydroxide and commercial elution buffer had a detrimental effect on the nylon NFs. Hence, neither of these well-known solutions should be used as stripping buffers with these NFs.

Glycine buffer pH 2.5 as stripping agent

In contrast, buffer containing glycine pH 2.5 was able to remove 70% of the total fixed antibody since the retained anti-

| Table 1: Percentage from original immunocapture system |
|----------------|----------|----------|----------|----------|----------|----------|
|                | Mean     | 100      | 5.1      | 60.9     | 40.9     | 65.5     | 35.3     |
| SEM            |          | 25.8     | 1.9      | 10.8     | 4.9      | 13.6     | 4.2      |
body after stripping treatment (group 2) was 30% compared to the total antibody bound in group 1 (Figure 5).

When the immunocapture system was rebuilt again after stripping (group 3), the amount of bound antibody (134%) was similar to that of total antibody bound before stripping (group 1, 100%) (Figure 5). It was also consistent with the BSA immunocapture results as the reconstituted immunocapture system (group 3) was able to bind the same amount of BSA (101.4%) as the immunocapture system before stripping (group 1, 100%) (Figure 6).

After glycine pH 2.5 treatment, when BSA was administered alone (group 4), only 35.9% of immunocaptured BSA was detected. The same percentage of antibody was detected after the stripping treatment (38.7%), both compared to group 1 (Figure 5 and Figure 6). When antibody was re-administered after stripping (group 5), the antibody was again fully bound (108.4% of bound antibody compared to group 1) (Figure 5), and BSA was immunocaptured in the same way (110%) (Figure 6). This suggests that only antibody, but not protein A/G, was eluted from the NFs.

In contrast to the previous treatments, no unspecific BSA binding was found with NFs treated with glycine pH 2.5 as both antibody and immunocaptured BSA showed the same percentage values compared to group 1.

Another interesting finding was that glycine buffer pH 2.5 damaged the bare NFs by rendering them unable to bind to the
immunocapture system (group 6). They were only able to bind 17.9% of the total antibody compared to group 1 (Figure 5). However, when protein A/G was anchored prior to treatment with glycine buffer pH 2.5 (group 5), the rebuilt immunocapture system showed the same rates of antibody immobilisation (108.4%) and immunocaptured BSA (110%) as group 1 (Figure 5 and Figure 6). This suggests that protein A/G protects the NFs from damage by the glycine buffer pH 2.5.

Discussion of nanofibre reusability
This study has shown that the effect of the pH value on protein A/G is very significant. A strong acid (pH 2.5) caused protein A/G to dissociate from the antibody, but not from the NFs. A higher pH value, such as ammonium buffer pH 11, caused the protein A/G to dissociate and/or not to anchor to the nylon nanofibres. The same results were obtained with the high-salinity commercial Ag/Ac elution buffer pH 6.6, which operates under near-neutral conditions but has a high salt content.

The structures of protein A/G and nylon and their interactions may explain all these results. On the one hand, protein A/G binds to the constant fraction (Fc) of the antibody by hydrophobic interactions through binding sites inside of its three-dimensional structure [21,22]. The polar side chains are located on the outside of the protein molecule, allowing the protein to form hydrogen bonds with nylon. On the other hand, nylon is a polyamide that contains amide groups and free amine groups at the ends of its polymer chains, as well as carboxyl groups.

**Figure 4**: RPE fluorescence of immunocaptured BSA. For each group, the RPE fluorescence data of the immunocaptured BSA, measured in RFU, are given as percentages relative to the reference group 1, \( n = 5–6 \). Stripping treatment with ammonium hydroxide buffer pH 11 was performed in all groups except group 1, which was used as the reference in the statistical analysis. One-way ANOVA followed by Newman–Keuls test. Difference from original immunocapture system fluorescence (group 1, reference group): *** \( p < 0.001 \).
These amide and amine groups provide excellent hydrogen bonding sites [23,24].

Regarding the binding of antibody to protein A/G, it has been described that this occurs at pH values between 5 and 8 because of hydrophobic interactions [21,22]. Acidic pH values below 5 cause protein A/G to separate from antibody, probably by imposing positive charges on amino acids with pKₐ values above 5, such as histidine, as described in Zarrineh et al. for the interaction between protein A and the Fc of antibody [25]. Our results are consistent with this; a strong acidic pH, such as glycine buffer pH 2.5, caused protein A/G to dissociate from antibody. Protein A/G was dissociated from nylon under basic pH conditions such as ammonium buffer pH 11. As the isoelectric point (pI) of protein A/G is 4.65, there is a higher percentage of acid groups, such as aspartic acid and glutamic acid. These aminoacids have carboxylic acid groups in their side chains, which lose protons at pH values higher than their pKₐ and become negatively charged as a result. In addition, nylon is negatively charged at basic pH [26]. This is understandable as nylon is a polyamide that contains not only many amide groups and free amine groups at the ends of its polymeric chains, but also a large number of carboxyl groups, more than amine groups, which give the nanofibres a negative charge in the basic pH range [23]. Therefore, basic pH levels such as pH 11, but...
Acidic pH, such as pH 2.5, does not alter the binding of protein A/G to nylon. However, bare nylon nanofibres were found to be altered by this treatment. This is understandable as polyamides, although containing both negative and positive centres, have amide and amine groups, which are protonated at acidic pH [23]. When protein A/G was administered prior to glycine buffer pH 2.5, no effect was observed as amide and amine groups will have previously formed hydrogen bonds with polar side chains on the outside of protein A/G [24].

In the case of the commercial Ag/Ac elution buffer pH 6.6 with high salt content, the high salt content, but not the pH value, may explain the results. The high salinity creates an environment of high ion concentration capable of interacting with any charge density group, disrupting the hydrophobic bonds between protein A/G and antibody and the hydrogen bonds between protein A/G and nylon, as well as the bare nylon nanofibres.

Results of stability study
As this system is designed to be used for the on-site detection of biological agents, we wanted to conduct a stability study using a potential biological warfare agent, that is, ricin. The NFs allow the immunocapture capability of the system to remain absolutely intact for one month without the use of any preservative. In contrast, a polypropylene microplate specifically designed to optimise an enzyme-linked immunosorbent assay showed a decreasing immunocapture capability such that seven days after the immunocapture system was assembled, only 44.6% of ricin was immunocaptured compared to the initial measurement result (day 0); after 30 days, only 18.1% was detected (Figure 7). Two-way ANOVA showed these differences to be statistically significant.

Discussion of stability study
As described by Feng et al. [27], hydrogen-bonded organic frameworks allow enzymes to diffuse into the pores, providing an additional layer of protection against denaturation factors. Since hydrogen bonds are formed between protein A/G and nylon, it is understandable that a three-dimensional nylon structure, such as the nanofibres, would provide more hydrogen bonds as attachment points than a planar surface, allowing the attached protein to be better protected.

Conclusion
In summary, NFs with protein A/G are capable to be reused in a new immunocapture system, as long as the stripping treatment is carried out with glycine buffer pH 2.5. After treatment with this buffer, protein A/G is separated from antibody but not from the NFs, and no damage in its antibody binding capability was found. This allows the system to be very cost-effective, not only because NFs can be used again, but also the previously anchored protein A/G. It reduces not only the cost, but also the time needed to provide a new immunocapture system ready to use. In addition, because the NFs protects the immunocapture system better than a planar surface specialised for anchoring antibodies, they allow the immunocapture system to extend its shelf life.

<table>
<thead>
<tr>
<th>Time</th>
<th>NF Mean</th>
<th>SF SEM</th>
<th>Treated polystyrene Mean</th>
<th>SF SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>100</td>
<td>12.3</td>
<td>100</td>
<td>5.0</td>
</tr>
<tr>
<td>7th day</td>
<td>94.72</td>
<td>7.2</td>
<td>44.6</td>
<td>7.5</td>
</tr>
<tr>
<td>30th day</td>
<td>119.9</td>
<td>21.0</td>
<td>18.1</td>
<td>4.0</td>
</tr>
<tr>
<td>90th day</td>
<td>0.3</td>
<td>6.5</td>
<td>3.7</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Figure 7: Fluorescence of ricin immunocaptured by the immunocapture system immobilised on both NFs and a specialised polypropylene ELISA microplate as function of the time, up to 90 days. Data are expressed as percentage of fluorescence of immunocaptured ricin on day 0, n = 4. Two-way ANOVA. Difference between the immunocapture system in NF and 96-well microplate, for each time: ***p < 0.001.
Experimental

Chemicals

PA6 was made by electrospinning by Tecnalia Research & Innovation, the composition of the ultrathin NFs was purchased from BASF (Ultramid® B24 N 03). The NF manufacturing procedure was described in previous publications [17-19,28,29]. The average diameter of the NFs was 23 ± 5.8 nm, determined using the “ImageJ” analysis software (Figure 8).

NFs were cut in 4 × 4 mm² samples to be placed and assayed in 96-well microplates. Nunc MaxiSorp® flat-bottom microplates were used in the stability assay. Ricin was obtained from Robert Koch Institute. FITC-labelling kit and LYNX Rapid HRP antibody conjugation kit were purchased from BioRad, Spain. The anti-ricin antibody is an in-house-manufactured mouse antibody, made in collaboration with the National Center for Biotechnology (CNB) – CSIC. 10-Acetyl-3,7-dihydroxyphenoxazine (ADHP, Ampliflu) was used as a fluorogenic substrate for horseradish peroxidase (HRP) (Sigma-Aldrich). BSA, from Sigma-Aldrich, labelled with RPE was selected as toxin surrogate. FITC-labelled sheep polyclonal antibody against BSA was purchased from Thermo Fisher Scientific Inc. The blocking buffer was phosphate-buffered saline (PBS) with casein (Pierce). Solvents and additives were purchased from Aldrich (Spain). PBS was purchased from Fisher Scientific.

Nanofiber reusability study

Immunocapture protocol

The immunocapture protocol used was published in 2018, as mentioned in the Introduction section [18]. It consisted of a well-oriented antibody immobilisation system containing the intermediate protein A/G. Briefly, NFs were placed in the microwells of a 96-well microplate, previously blocked with PBS–casein. In order to achieve a well-oriented antibody immobilisation, protein A/G (10 µL 100 µg/mL in PBS) was added to each NF sample and incubated overnight at 4 °C, followed by a blocking step with PBS–casein. Then, a FITC-labelled antibody against BSA was immobilized on the surface of the NFs containing protein A/G 1 h of incubation. Then, RPE-labelled BSA (10 µL 100 µg/mL in blocking buffer) was immunocaptured by the anchored antibodies over a period of 1 h. Washing steps were carried out between each step in order to remove non-linked excess reagents. The fluorescence signals were measured using a Gemini XPS Microplate Reader (Molecular Devices) in RFU.

Anchored antibody was measured as FITC-fluorescence (λ_emission = 490 nm and λ_excitation = 521 nm) after incubation and subsequent wash, divided by the FITC fluorescence obtained just before antibody incubation (autofluorescence of the system).

Immunocaptured BSA was measured as RPE fluorescence (λ_emission = 495 nm and λ_excitation = 521 nm) after incubation and subsequent wash, divided by the RPE autofluorescence obtained just before antibody incubation.

Since a lot of handling is required, BSA was used as a toxin surrogate in “Nanofibre reusability study” because of safety and economic considerations.

Stripping treatments

Since we wanted to evaluate the role played by the pH value, three different pH buffers from acidic to basic were assayed. We used a Thermo Scientific™ Pierce™ Gentle Ag/Ab elution buffer pH 6.6, a glycine buffer pH 2.5 containing 200 mM glycine in PBS, and an ammonium hydroxide buffer pH 11 containing 1 N NH₄OH in PBS (the latter two chemicals were purchased from Sigma-Aldrich).

The stripping protocol using any buffer was as follows: Stripping buffer was added (200 µL per NF sample) and incubated at room temperature for 10 min two times. The stripping buffer was removed from the nanofibers, and the NFs were washed with PBS (adding and incubating for 10 min two times). The two stripping buffer steps were repeated and three 5 min PBS wash steps took place after them (adapted from abcam stripping protocols [30]).

Reconstructing of the immunocapture system

The immunocapture systems were rebuilt as described above. In order to study how each treatment affects both immunocapture system and nanofibers, several groups were assayed: Group 1: complete immunocapture system (protein A/G + antibody-FITC + BSA-RPE) without stripping treatment (group 1). Group 2:
complete immunocapture system (protein A/G + antibody-FITC + BSA-RPE) with stripping treatment. **Group 3:** complete immunocapture system (protein A/G + antibody-FITC + BSA-RPE), then stripping treatment and complete rebuild of the immunocapture system (protein A/G + antibody-FITC + BSA-RPE) afterward. **Group 4:** immunocapture system without BSA-RPE, then stripping treatment and only BSA-RPE added afterward. **Group 5:** only protein A/G anchored to NFs, then stripping treatment and only antibody-FITC incubation and BSA-RPE added afterward. **Group 6:** only bare NFs undergoing stripping treatment and complete rebuild of the immunocapture system (protein A/G + antibody-FITC + BSA-RPE) afterward.

Fluorescence of both anchored FITC-antibody and immunocaptured BSA-RPE was measured as described above. Results are shown as percentage fluorescence of the complete immunocapture system compared to the group 1, which is the 100% value. Data were statistically analysed by two two-way analysis of variance (ANOVA) using GraphPad Prims 5 Software.

**Stability study**
Since less handling is required in this study, ricin is used as toxin instead of a surrogate as BSA.

**Immunocapture system in stability study**
The immunocapture system was similar as one described above. Briefly, NFs were placed in the microwells of a 96-well microplate, previously blocked with PBS–casein. Protein A/G (10 µL 100 µg/mL in PBS) was added to each NF sample surface and incubated over night at 4 °C, followed by a blocking step with PBS–casein. The control planar surface group was incubated with protein A/G overnight and then blocked with PBS–casein. Since only the immunocapture capability was measured in this study, nonlabelled in-house antibody (10 µL 500 µg/mL) against ricin was incubated for 1 h at room temperature. Then, biotin-labelled ricin (1 µL 1 mg/mL in blocking buffer) was immunocaptured by the anchored antibodies over 1 h of incubation. Biotin-ricin was added to both NF and microplate immunocapture systems at different times: day 0 (immediately after antibody anchoring; it is considered the reference value) and 7 days, 30 days, and 90 days after antibody anchoring; it is considered the reference value.

**Considerations**
(1) The samples must be dissolved in buffer with a physiological pH value before testing them. (2) The sensing method used was fluorescence as it is a simple method that does not require any additional steps for its determination. However, even though the aim of the study was to evaluate the reusability and stability of NFs, the sensitivity of the system could be improved by using another more accurate sensing system. (3) Because of the pore size of the nanofibres, they cannot be used for the detection of bacteria, rickettsiae, or fungi (i.e., they cannot be used for the detection of prokaryotic or eukaryotic cells). They could, therefore, be used for the determination of exogenously produced biotoxins and virulence factors, as well as for the detection of viruses and biomarkers in clinical samples (e.g., hormones and biomolecules). (4) The data could be generalised not only for the measurement of warfare agents, but also for the diagnosis of water and food contamination and for the clinical diagnosis of infectious agents and biomarkers.

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We would like to thank the whole team at Nieves Murillo of TECNALIA – Industry and Transport Division for providing the nylon NFs.

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**References**
Vinorelbine-loaded multifunctional magnetic nanoparticles as anticancer drug delivery systems: synthesis, characterization, and in vitro release study

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Abstract

In this study, a multifunctional therapeutic agent combining chemotherapy and photothermal therapy on a single platform has been developed in the form of vinorelbine-loaded polydopamine-coated iron oxide nanoparticles. Vinorelbine (VNB) is loaded on the surface of iron oxide nanoparticles produced by a solvothermal technique after coating with polydopamine (PDA) with varying weight ratios as a result of dopamine polymerisation and covalent bonding of thiol-polyethylene glycol (SH-PEG). The VNB/PDA/Fe₃O₄ nanoparticles have a saturation magnetisation value of 60.40 emu/g in vibrating sample magnetometry, which proves their magnetisation. Vinorelbine, which is used as an effective cancer therapy agent, is included in the nanocomposite structure, and in vitro drug release studies under different pH conditions (pH 5.5 and 7.4) and photothermal activity at 808 nm NIR laser irradiation are investigated. The comprehensive integration of precise multifunctional nanoparticles design, magnetic response, and controlled drug release with photothermal effect brings a different perspective to advanced cancer treatment research.

Introduction

Cancer is a widespread condition characterized by the uncontrolled proliferation of aberrant cells, which can spread to diverse body regions, encompassing over a hundred distinct forms [1,2]. Current cancer treatments lack a complete approach, as they mostly rely on radiotherapy, chemotherapy, immunotherapy, and surgery in clinical environments [3]. While these methodologies provide therapeutic benefits, they also contribute to cancer progression by inducing cytotoxicity in
healthy cells and weakening the immune system, rendering individuals more vulnerable to other ailments [4,5]. There is a must to develop alternative multifunctional methodologies or intelligent drug delivery systems to formulate more effective cancer treatments, thereby addressing the current limitations encountered within this field of study. Functional nanostructures have been designed to mitigate potential harm to healthy tissue caused by these techniques [6]. Additionally, they facilitate passive targeting and offer multimodal tumor therapy.

In recent years, the use of nanotechnology-based cancer drugs has emerged as a promising alternative treatment approach. Utilizing various nanostructures as specific vehicles for drug delivery enhances efficacy and pharmacokinetic properties of anticancer drugs while mitigating the adverse effects of large dosage administration [6,7]. Additionally, it offers several advantages, such as controlled release, targeted drug delivery, and improved stability [8]. Moreover, nanoscale drug delivery systems hold great promise for specific cancer treatments, as they increase permeability and retention effect in solid tumors, enabling precise application to the targeted cells. Various structures such as silica-based conjugates, inorganic polymers, ceramic nanomaterials, gold, iron oxide, and noble metal nanoparticles have been utilized [9,10]. Among the nanostructures employed, particular emphasis has been placed on iron oxide (Fe₃O₄) nanoparticles. The biocompatibility and low toxicity of Fe₃O₄ nanoparticles have garnered significant attention in magnetic drug delivery for cancer diagnosis and treatment, primarily because of their magnetic properties [11,12]. The crystal structure of Fe₃O₄ nanoparticles can be tailored to allow for precise control, and these nanostructures find utility in various production processes. Magnetite nanoparticles exhibit superparamagnetic behavior due to the negligible energy barrier in the hysteresis of the particles’ magnetization cycle, as Bloch and Neel theorized [11,13]. Superparamagnetic iron oxide nanoparticles for drug delivery, diagnosis, and cancer therapy have gained wider acceptance in biomedical applications [14]. They have received notable attention in clinical applications such as early disease diagnosis (e.g., cancer, diabetes, and atherosclerosis), magnetic resonance imaging (MRI), targeted drug delivery, photothermal therapy, gene therapy, and molecular and cellular monitoring [15,16]. Photothermal therapy (PTT), a treatment in which nanostructures are used, induces drug release or damages tumor cells with the heat produced by nanostructures under NIR laser irradiation [17,18]. Compared to traditional treatments, photothermal therapy allows for increased drug release and is less cytotoxic to healthy tissues [19]. It is a minimally invasive technique that offers the advantage of rapid recovery [20]. Many well-designed agents have been developed for photothermal therapy, including carbon, metal, and organic nanocomposites [21]. Due to their superparamagnetic and heating potential, Fe₃O₄ nanoparticles have recently garnered attention, particularly in photothermal therapy research. Dopamine (DA) is a neurotransmitter naturally occurring in the brain and can spontaneously polymerize into polydopamine (PDA) under alkaline conditions without oxidants [22]. Polydopamine can form a coating with biocompatibility advantages, achieving up to 40% photothermal conversion efficiency, nanoscale dimensions, and customizable morphology [23,24]. Additionally, the photothermal conversion efficiency of PDA, PDA concentration, reaction time, and PDA thickness can be adjusted. Importantly, PDA exhibits a 40% photothermal conversion rate with excellent photothermal stability, indicating its significant potential as a NIR laser-driven photothermal agent [25]. However, it is challenging to completely eradicate solid tumors using PTT alone because of light scattering and limited absorption in tumor tissues. For this purpose, various modifications have been employed for passive tumor targeting. PEGylation, which involves the use of poly(ethylene glycol) (PEG) polymer, is a widely used modification method to improve passive tumor targeting and retention [26-28]. In a study presented in the literature, PEGylation was used to impart passive tumor targeting properties to PDA nanoparticles. In vivo experiments where the synthesized nanostructure was exposed to NIR light, SN38-loaded nanoparticles effectively suppressed tumor growth chemotherapeutically and photothermally [29]. This promising result highlights the potential of the PEGylation of PDA nanoparticles for advanced cancer treatment strategies. Vinorelbine (VNB), a chemotherapeutic agent, has seen significant clinical use in the treatment of lung cancer and advanced breast cancer [30]. VNB affects the continuous mitotic division in cancer cells, thereby impeding uncontrolled growth. By binding to microtubules, VNB exerts an inhibitory effect on cancer cell growth, slowing their proliferation and disrupting mitotic regulation, leading to the stimulation of the tumor suppressor gene p53 and the inhibition or inactivation of various signaling pathways [31,32]. Its widespread adoption in medicine can be attributed to its strong therapeutic efficacy. The application of vinorelbine tartrate is limited because of its dose-related toxicity to the nervous, pulmonary, and gastrointestinal systems and reduced absorption when taken orally [33]. Encapsulation studies specifically aim to create a controlled drug delivery system to reduce existing side effects of cancer drugs or to significantly increase clinical compliance.

Zhao et al. synthesized vinorelbine-loaded and RGD-functionalized polydopamine-coated Fe₃O₄ superparticles via thermal decomposition [34]. Our study utilizes a solvothermal method to synthesize nanostructures with a spherical morphology and a size of 18 nm. After coating with PDA at different ratios, the size reaches up to 28, 61, and 225 nm. Another point is that PEGylation has been applied using SH-PEG polymer to en-
hance biocompatibility. Notably, our study demonstrates a significantly higher drug loading efficiency of 98%, indicating the superior efficacy of our synthesis method. Moreover, our drug-loaded nanostructures exhibit a saturation magnetization of $M_s = 60.40 \text{ emu/g}$, highlighting enhanced magnetic properties compared to the cited study. This indicates that the nanostructure can be strongly manipulated under an external magnetic field. This finding is crucial for future studies on magnetic field-guided drug release and tumour treatment. Particularly, our research also investigates the effect of varying polymer ratios on drug release kinetics and photothermal efficiency, which was not addressed in the abovementioned paper. It was demonstrated through this study that as the amount of PDA polymer increased, both photothermal heating efficiency and drug release decreased, while the drug release rate increased when photothermal heating was applied. Fe$_3$O$_4$ nanoparticles with adjustable magnetic properties and appropriate sizes exhibited controlled drug release capabilities. Thus, a controlled drug delivery system was established using VNB/PDA/Fe$_3$O$_4$ NPs, which exhibited high release at the tumor microenvironment pH 5.5 for potential application in cancer treatment. The impact of polymer thickness on drug release was also determined.

Consequently, our study represents a novel contribution to the field by investigating the impact of polymer thickness on drug release, offering enhanced drug loading efficiency, improved magnetic properties, and pH-responsive drug release kinetics.

Materials and Methods

Materials

The chemicals used in nanoparticle synthesis, namely iron(III) chloride hexahydrate (FeCl$_3$·6H$_2$O, $M_w = 270.30 \text{ g/mol}$), iron(II) chloride tetrahydrate (FeCl$_2$·4H$_2$O, $M_w = 198.81 \text{ g/mol}$), dopamine hydrochloride ($M_w = 189.64 \text{ g/mol}$), tris(hydroxymethyl)aminomethane hydrochloride, (Tris-HCl, $M_w = 157.60 \text{ g/mol}$), and thiol-polyethylene glycol (SH-PEG, $M_w = 189.64 \text{ g/mol}$), were removed by washing with distilled water. The PEGylation of Fe$_3$O$_4$ nanoparticles with SH-PEG was achieved through the Michael addition reaction, involving the thiol and carbonyl groups present in the PDA polymer. Approximately 5 mL of ammonium hydroxide (NH$_4$OH, 28–30%), was added, and the mixture was stirred at room temperature for 15 h at 1000 rpm. Following this stage, the obtained PDA-coated Fe$_3$O$_4$ NPs were separated from the solution using a magnet. Subsequently, unreacted material was removed by washing three times with distilled water, and the PDA/Fe$_3$O$_4$ NPs were dried in a vacuum oven at 55 °C.

Surface functionalization of PDA/Fe$_3$O$_4$ nanoparticles with SH-PEG

For the surface modification process with SH-PEG, 50 mg of PDA/Fe$_3$O$_4$ NPs were added to 50 mL of Tris-HCl solution. Then, 100 mg of thiol-polyethylene glycol was added to the prepared mixture to homogenize the solution. Subsequently, 0.2 mL of ammonium hydroxide (NH$_4$OH, 28–30%), PEGylation PDA/Fe$_3$O$_4$ NPs were separated from the solution using a magnet, and unreacted particles were removed by washing with distilled water. The PEGylation PDA/Fe$_3$O$_4$ NPs were then dried in a vacuum oven at 45 °C.

The PDA-coated Fe$_3$O$_4$ nanoparticles were modified with SH-PEG to facilitate their accumulation in tumour regions. In similar studies, the conjugation of SH-PEG onto the surface of PDA polymer was achieved through the Michael addition reaction, involving the thiol and carbonyl groups present in the SH-PEG structure [37,38].

Vinorelbine loading on PDA/Fe$_3$O$_4$ nanoparticles

For loading vinorelbine tartrate into the nanostructure, 25 mg of PEGylation PDA/Fe$_3$O$_4$ nanoparticles (1 mg/mL) were combined with 25 mL of phosphate solution (pH 8.5). The purpose of this mixture was to facilitate the loading of vinorelbine tartrate into the PDA/Fe$_3$O$_4$ content. Subsequently, 25 mg of VNB was added to the prepared mixture, and the solution was thoroughly mixed for 5 h. The resulting nanostructure was separated with the assistance of a magnet, followed by three thorough rinses with distilled water. All wash supernatants were collected to measure the VNB loading content based on UV–vis spectrophotometry. The resulting nanostructure underwent...
vacuum drying at 45 °C. The loading entrapment efficiency (%EE) [39] of VNB into Fe₃O₄ NPs was calculated using Equation 1; it was found that the entrapment efficiency was approximately 98%.

\[
EE(\%) = \frac{\text{initial drug amount} - \text{unentrapped drug}}{\text{total drug amount}} \times 100 \tag{1}
\]

**Standard curve of vinorelbine**
The calibration curve for the time-dependent release of VNB was generated by preparing VNB solutions at concentrations of 0, 20, 40, 60, 80, 100, 200, and 400 µg/mL. These solutions were then placed in quartz cuvettes, and absorbance readings were taken using the UV–vis spectrophotometer at a wavelength of 268 nm [40]. The absorbance values obtained were utilized to construct the calibration curve.

**Determination of photothermal stability and efficiency**
Fe₃O₄ NPs, PDA/Fe₃O₄ NPs, and VNB/PDA/Fe₃O₄ NPs (at a concentration of 0.1 mg/mL and in a total volume of 1 mL) were exposed to 808 nm (1 W/cm²) NIR laser irradiation for a duration of 5 min. PBS was used as a control. The temperature changes of the NP solutions were recorded using an infrared thermal imaging camera. Additionally, the photothermal stability of both PDA/Fe₃O₄ NPs and VNB/PDA/Fe₃O₄ NPs (at a concentration of 0.1 mg/mL and in a total volume of 1 mL) was assessed through a 5 min interaction with an 808 nm (1 W/cm²) NIR laser followed by a cooling process, for four cycles [41].

**Determination of vinorelbine drug release**
VNB/PDA/Fe₃O₄ NPs (at ratios of 1:1, 2:1, and 4:1) were placed into dialysis capsules at a concentration of 1 mg/mL. Subsequently, each prepared dialysis capsule was placed in 100 mL phosphate solution at pH 5.5 and 7.4 [30]. The experiment was conducted at 37 °C with a shaking speed of 150 rpm. The experiment involved obtaining a 1 mL sample at specified time intervals (0.5, 1, 2, 3, 4, 5, 6, 12, 24, 30, 36, 48, and 50 h). The sample was then analyzed using a UV–vis spectrophotometer. The mean values of the results obtained in triplicate were taken. The concentration of the drug release was calculated using Equation 2 [42] with the calibration curve for VNB.

\[
\text{concentration of drug (µg/mL)} = (\text{slope} \times \text{absorbance}) \pm \text{intercept} \tag{2}
\]

In the in vitro dissolution test, the drug release (DR) was determined using Equation 3.

\[
\text{DR (mg/mL)} = \frac{\text{(concentration} \times \text{dissolution bath volume)}}{\text{1000 \times \text{dilution factor}}} \tag{3}
\]

The cumulative percentage of drug release (CPR %) was determined using Equation 4 [42] separately for pH 5.5 and 7.4. Furthermore, the cumulative drug release percentages of VNB/PDA/Fe₃O₄ NPs prepared with different ratios were compared to investigate the effect of the PDA ratio on VNB drug release.

\[
\text{CPR (\%) = } \frac{\text{the volume of samples withdrawn (mL)} \times P_{t} - P_{t-1}}{\text{bath volume (v)}}, \tag{4}
\]

where \(P_t\) is the percentage release at time \(t\) and \(P_{t-1}\) is the previous percentage release.

**Determination of photothermal-responsive drug release**
The dialysis capsules were exposed to an 808 nm (1 W/cm²) NIR laser for 5 min at specific time points (15, 30, 45, 60, 120, 240, and 300 min) to assess the impact of laser irradiation on drug release. The drug content in VNB/PDA/Fe₃O₄ NPs (at ratios of 1:1, 2:1, and 4:1) and the cumulative drug release were determined by calculating according to Equations 2–4 utilizing the absorbance values obtained from UV–vis spectrophotometer and calibration curves.

**Characterization**
The morphology of the synthesized nanoparticles was determined with a high-resolution analytical electron microscope (FE-SEM, Thermo Scientific, Apreo 2S LoVac) and a scanning transmission electron microscope (STEM, Phillips XL, 30 ESEM-FEG/EDAX) operating at 120 kV acceleration voltage. The structure of the nanoparticles was analyzed by X-ray diffraction (XRD, PANalytical, Xpert Pro) using Cu Kα radiation (\(\lambda = 0.15418\) nm) in a 2θ range of 10° to 90°. Fourier-transform infrared (FTIR, Thermo, Nicolet Is 10) spectra of the nanostructures were obtained in the range of 4000–400 cm⁻¹.

The amounts of released drug were obtained using a UV–vis spectrophotometer (Shimadzu Scientific Instruments, UV-1800) at a wavelength of 268 nm. Magnetic properties of nanoparticles were evaluated by vibration sample magnetometry (VSM, Lake Shore, Model 7410) using field-induced magnetization measurements at 298 K. The average diameters of nanoparticles were determined using ImageJ (US National Institute of Health, Bethesda, MD, USA). OriginPro 8.5 (OriginLab, MA, USA).
Results and Discussion
Characterization of Fe₃O₄ nanoparticles

The synthesis of Fe₃O₄ NPs was carried out using a solvothermal technique in a stainless steel reactor at 200 °C for 6 h. According to the results of FE-SEM and STEM examinations, the Fe₃O₄ NPs are spherical, as depicted in Figure 1a–c. When examining the STEM size distribution, it was observed that Fe₃O₄ NPs were efficiently synthesized with an average size of 18 nm.

The Fe₃O₄ NPs consist of 99.9% magnetite and have a cubic reverse spinel structure. Magnetite exhibits a spinel crystal structure resulting in a face-centered cubic arrangement in which oxygen atoms are positioned opposite the other constituent atoms. The Fe₃O₄ NP (311) reflection shows a significantly wide full width at half maximum, indicating the presence of ultrafine particles and a small crystal size. The crystal size was determined using the Scherrer equation (Equation 5) [43] applied to the most prominent diffraction peaks of Fe₃O₄ NPs.

\[ D = \frac{K \cdot \lambda}{\beta \cdot \cos \theta} \]

Equation 5 shows the relationship between peak broadening and particle size in X-ray analysis. In this equation, the symbols \( D \), \( K \), \( \lambda \), \( \beta \), and \( \theta \) represent the particle size, Scherrer shape factor (here 0.89), X-ray wavelength (0.15418 nm), half-maximum width, and diffraction angle, respectively [43]. Using the X-ray diffraction (XRD) spectrum and Equation 5, the particle size of Fe₃O₄ NPs was calculated and determined to be 18 nm on average.

X-ray patterns showing the distribution of Fe₃O₄ NPs in their uncoated state are shown in Figure 2a. XRD analysis reveals the presence of seven distinct peaks at 30.13°, 35.48°, 43.12°, 53.6°, 56.08°, 62.7°, and 73.92°. These peaks can be assigned to...
the corresponding crystallographic planes of magnetite Fe$_3$O$_4$: (220), (311), (400), (422), (511), (440), and (533), respectively. Fe$_3$O$_4$ NPs exhibit a peak consistent with the data obtained for the reference ICDD no. 19-629 [43].

The FTIR spectra presented in Figure 2b show the characteristic peak associated with the Fe–O bond of Fe$_3$O$_4$ NPs at a wavenumber of 580 cm$^{-1}$. The peak detected at a wavenumber of 1420 cm$^{-1}$ was attributed to –OH groups in Fe$_3$O$_4$ NPs [44]. A vibrating sample magnetometer (VSM) was used to investigate the magnetic properties of the Fe$_3$O$_4$ NPs [45]. Various factors such as the crystal structure of the material, dimensions, morphology, and density of crystal defects significantly affect the magnetic properties [46]. The saturation magnetization ($M_s$) values of NPs measured at 298 K using a VSM are given in Figure 2c. The values obtained for saturation magnetization ($M_s$), coercivity ($H_c$), and residual magnetization ($M_r$) were determined as 67.72 emu/g, 63.37 Oe, and 4.39 emu/g, respectively.

Characterization of polydopamine coating, PEGylation, and drug loading modifications of Fe$_3$O$_4$ nanoparticles

The Fe$_3$O$_4$ NPs were synthesized using a solvothermal method. Subsequently, Fe$_3$O$_4$ NPs were coated with PDA in different ratios. The coating process involved the use of PDA in ratios of 1:1, 2:1, and 4:1, respectively. PDA can undergo polymerization resulting in the adsorption onto the surface of the negatively charged Fe$_3$O$_4$ NPs [22]. During this process in an alkaline environment, PDA polymerizes into its oxide form. As a result, the nanostructure undergoes coating with PDA [47]. The average distribution of PDA coating sizes and thicknesses was determined using FE-SEM size analysis.

In Figure 1c, the average size of bare Fe$_3$O$_4$ NPs was 18 nm, whereas in Figure 3a, after PDA coating (1:1 ratio), the average size of Fe$_3$O$_4$ NPs was 28 nm. Hence, it is postulated that Fe$_3$O$_4$ NPs have been subjected to 10 nm PDA coating. Figure 3b shows the PDA/Fe$_3$O$_4$ NPs with 2:1 ratio demon-
Figure 3: FESEM images of (a) PDA/Fe₃O₄ NPs (1:1), (b) PDA/Fe₃O₄ NPs (2:1), and (c) PDA/Fe₃O₄ NPs (4:1), all 250,000× magnification. (d) FTIR spectra of Fe₃O₄ NPs and PDA/Fe₃O₄ NPs (1:1, 2:1, and 4:1 ratio).

strating an average size of 61 nm and an average coating thickness of 43 nm. In a similar vein, it can be observed from Figure 3c that the PDA/Fe₃O₄ NPs (4:1 ratio) exhibit an average size of 225 nm, corresponding to a coating thickness of 103.5 nm. The FTIR spectra of Fe₃O₄ NPs and PDA/Fe₃O₄ NPs (1:1, 2:1, and 4:1) are given in Figure 3d. The absorption peaks at 587 and 1620 cm⁻¹ are indicative of Fe₃O₄ NPs. These results are in line with a study by Xue and co-workers [45]. The broad absorption bands in the 1700–1000 cm⁻¹ range suggest the presence of aromatic rings and phenolic compounds in PDA. These bands demonstrate the effective coating with PDA, as illustrated in Figure 3d.

A study by Feng et al. noted a peak at 1259 cm⁻¹ in the IR spectra of PDA/Fe₃O₄ NPs, attributed to the extension vibration of the C–O band. The obtained results are supported by the resemblance to the peak observed at 1221 cm⁻¹ in this observation. Furthermore, the peaks observed at 1520 and 1595 cm⁻¹ can be attributed to the stretching vibration of C–O units, which is further supported by the peak at 1221 cm⁻¹ [48].

Studies in the literature have demonstrated that the coating of iron oxide nanoparticles, commonly employed in creating multifunctional particles with the capability of passive targeting in magnetic fields for photothermal cancer therapy, with PDA holds great promise for future applications. Therefore, surface modification with PDA is recognized as a favorable alternative for enhancing the biocompatibility of non-biodegradable substances.

A study focused on examining the binding of PEG to PDA/Fe₃O₄ NPs and the resulting chemical structure using FTIR
spectroscopy. According to the FTIR analysis results, the peak at 585 cm\(^{-1}\) in the spectrum corresponds to the vibration associated with the Fe–O bond in magnetic nanoparticles [49]. The peak observed at 3400 cm\(^{-1}\) can be attributed to the vibration associated with stretching hydroxy (–OH) groups in Fe\(_3\)O\(_4\) NPs (Figure 4a). FTIR analysis revealed that the peaks observed at 1150 and 2890 cm\(^{-1}\) correspond to the vibrations associated with stretching C–O–C and C–H groups in SH-PEG, respectively [50]. The presence of band structures at 1500 and 1000 cm\(^{-1}\) in the FTIR analysis of SH-PEG provides evidence for the surface modification of PDA/Fe\(_3\)O\(_4\) NPs with SH-PEG. The FTIR analysis results indicate the successful functionalization of PDA/Fe\(_3\)O\(_4\) NPs with SH-PEG molecules. This will enable the passively targeted delivery of the created nanoplatform to tumor tissues and enhance biocompatibility. Similar studies have described conjugated PEG–iron oxide nanoparticles as multifunctional nanotherapeutic agents for passive targeting of tumors [49,50]. Additionally, in the literature, it has been demonstrated that multifunctional PEGylated magnetic nanoparticles coated with polydopamine (PDA) exhibit strong near-infrared absorption because of the PDA layer and have the ability to deliver drugs under a magnetic field owing to their superparamagnetism [51].

During the drug loading studies, the anticancer drug vinorelbine was incorporated in the structure of PDA/Fe\(_3\)O\(_4\) NPs during the polymerization of dopamine. It was observed that a significant portion of VNB in VNB/PDA/Fe\(_3\)O\(_4\) NPs was absorbed within the polymer shell, while a small portion was retained on the surface [52]. According to FTIR analyses, drug-related features are visible in the nanostructures as N–H peaks located at 3500–3000 cm\(^{-1}\) (Figure 4b). This result demonstrates the effective incorporation of VNB into the nanostructure. As a result, the VNB compound exhibits a prominent peak attributed to the presence of a carbon–carbon (C–C) group at 1573 cm\(^{-1}\) and a nitrogen–hydrogen (N–H) group in the range of 3500–3000 cm\(^{-1}\) [53]. The FTIR spectrum of VNB/PDA/ PDA/Fe\(_3\)O\(_4\) NPs displays all PDA, SH-PEG, and VNB peaks, indicating the successful formation of a core–shell structure containing these three components.

![Figure 4:](image.png)

*Figure 4:* (a) FTIR spectra for Fe\(_3\)O\(_4\) NPs and PDA/Fe\(_3\)O\(_4\) NPs. (b) FTIR spectra for VNB and VNB/ PDA/Fe\(_3\)O\(_4\) NPs. (c) Hysteresis loops for Fe\(_3\)O\(_4\) NPs, PDA/Fe\(_3\)O\(_4\), and VNB/PDA/Fe\(_3\)O\(_4\) NPs.
According to the VSM analysis, the saturation magnetization of Fe₃O₄ NPs was 67.72 emu/g; PDA/Fe₃O₄ NPs had a saturation magnetization of 65.62 emu/g; VNB/PDA/Fe₃O₄ NPs showed a saturation magnetization of 60.40 emu/g, as shown in Figure 4c. The observed decrease in magnetization is commonly attributed to the polymer coating on the surface of the magnetic nanoparticles [49]. Based on the findings from VSM, the nanoparticles exhibit high magnetization [49,54]. The magnetic properties of VNB/PDA/Fe₃O₄ NPs can be attributed to the structural arrangement of Fe₃O₄ within the nanoparticles. A magnetic field can enhance the dispersion of VNB/PDA/Fe₃O₄ NPs in an aqueous solution, showing promising prospects for use in magnetically targeted therapy.

**Determination of photothermal stability and efficiency**

To evaluate the photothermal performance of the nanostructures, the NPs were irradiated with an 808 nm laser at a power density of 1 W/cm² for 5 min. A slight increase in temperature was observed in the phosphate-buffered saline (PBS, control) solution. As shown in Figure 5b, when exposed to NIR laser, the temperature of the Fe₃O₄ NP solution reached a maximum of 37.6 °C.

In contrast, Figure 5b(b,d) illustrates a rapid increase in the temperature of PDA/Fe₃O₄ NPs, reaching a peak at 47.6 °C after 5 min. This swift temperature rise is likely attributed to the enhanced stability of PDA in PDA/Fe₃O₄ NPs and its higher NIR absorption capabilities. The temperature of VNB/PDA/Fe₃O₄ NPs (at ratios of 1:1, 2:1, and 4:1) varied between 45.3 °C and 45.9 °C after 5 min of laser irradiation. All drug-loaded nanostructures reached a heating temperature of 40 °C after 3 min and did not exceed a maximum temperature of 46 °C. These findings indicate a promising potential for applying these nanostructures in photothermal therapy. Figure 5c presents the results of the photothermal efficiency study for PDA/Fe₃O₄ and VNB/PDA/Fe₃O₄ NPs. Following four cycles of NIR laser irradiation, the photothermal stability of the NPs was maintained.

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**Figure 5:** (a) Infrared thermal camera images of NPs at ratios of 1:1, 2:1, and 4:1, respectively, after 808 nm laser (1 W/cm²) irradiation: (a, d) VNB/PDA/Fe₃O₄ NPs at a 1:1 ratio, (b, e) VNB/PDA/Fe₃O₄ NPs at a 2:1 ratio, and (c, f) VNB/PDA/Fe₃O₄ NPs at a 4:1 ratio. (b) Infrared thermal camera images of (a, c) Fe₃O₄ NPs and (b, d) PDA/Fe₃O₄ NPs after 808 nm laser (1 W/cm²) irradiation. (c) Temperature changes during laser interaction. (d) Cyclically repeated temperature changes.
Table 1: Cumulative drug release.

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<td>67.2743</td>
<td>64.12168</td>
<td>55.09965</td>
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NIR irradiation resulted in enhanced drug release due to the heightened temperature induced by the thermosensitive Fe$_3$O$_4$ NPs. Furthermore, the enhanced heating due to the photothermal properties of PDA facilitated the separation of VNB from the structure [45]. As shown in Figure 5c, it is worth noting that the temperature of PDA/Fe$_3$O$_4$ NPs increased from 25 to 45 °C in 3 min following the NIR irradiation and reached 47.6 °C after 5 min.

Regarding the drug release from laser-irradiated VNB/PDA/Fe$_3$O$_4$ NPs (2:1), there was a 68% increase in drug release within the initial 12 h, with 81% of the drug being released within 50 min at pH 7.4; 64% release was observed after 50 h. For VNB/PDA/Fe$_3$O$_4$ (4:1), possessing the highest PDA ratio, the drug release was 75% at pH 5.5 and 55% at pH 7.4. The observed drug release rates of VNB/PDA/Fe$_3$O$_4$ NPs (specifically, at ratios 2:1 and 4:1) reaching a maximum of 78% after 300 min is intriguing (Table 2).

One possible explanation for this phenomenon lies in the thickness of the PDA coating. It is plausible to consider that as the PDA layer becomes thicker, it may pose a barrier to efficient heating of the Fe$_3$O$_4$ core. This could result in a delayed release compared to formulations with a thinner PDA layer.

Moreover, it’s worth noting that a thicker polymer layer may impede surface erosion. This aspect is crucial in drug release, as it can lead to a slower, more controlled release of the encapsulated drug [55]. Thus, the thickness of the PDA coating emerges as a pivotal factor influencing the release dynamics of the

### Table 2: Laser interaction and cumulative drug release for VNB/PDA/Fe$_3$O$_4$ NPs.

<table>
<thead>
<tr>
<th>Time (mn)</th>
<th>pH 5.5</th>
<th>pH 7.4</th>
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<tr>
<td></td>
<td>1:1</td>
<td>2:1</td>
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<td>78.52115</td>
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</table>
loaded drug [56]. Another critical aspect that warrants attention is the potential impact of different pH values on the oxidative capacity of the coating material polydopamine. This coating, formed through the polymerization of dopamine, plays a pivotal role in the drug delivery system. The variations in pH values can potentially modulate the chemical environment in which the polymerization occurs [57].

Consequently, pH alterations may induce changes in the surface charge of the nanoparticles. This could profoundly affect the drug binding capacity of the nanoparticles and implies that the nanoparticles may exhibit varying affinities for the drug molecule at different pH values. Hence, the observed differences in drug release profiles between pH 5.5 and 7.4 can be plausibly attributed to these pH-dependent interactions [57]. Factors such as pH value, coating material properties, coating thickness, and drug binding capacity significantly influence drug release [58].

Based on the presented findings, it can be concluded that polymer thickness and NIR laser irradiation affect the drug release process. When externally applied, NIR laser irradiation can facilitate the release of VNB from VNB/PDA/Fe₃O₄ NPs and induce a photothermal interaction at the tumor site. The synthesized VNB/PDA/Fe₃O₄ NPs promise for effective photothermal therapy, magnetic targeting, MRI imaging, and chemotherapeutic capabilities in future studies.

Conclusion
In this study, we successfully synthesized VNB/PDA/Fe₃O₄ NPs with combined photothermal therapy and chemotherapy functionalities using a solvothermal method. The incorporation of PDA into the fabricated nanostructures imparts several advantages for cancer therapy and controlled drug release systems because of its robust structural and physicochemical properties. Additionally, aside from enhancing photothermal therapy capabilities, the PDA shell mitigates nanomaterial toxicity while increasing biocompatibility. The strategic integration of PEGylation into tumor-targeted drug delivery systems significantly amplifies passive tumor targeting and retention through the enhanced permeability and retention effect, thereby enhancing its efficacy in cancer treatment. Furthermore, our findings underscore the pivotal roles played by polymer thickness, the acidic tumor microenvironment, and NIR laser irradiation in the drug release process. Notably, the application of a NIR laser in conjunction with the acidic tumor microenvironment triggers the controlled release of VNB. When combined with laser-induced photothermal therapy, this results in effective tumor elimination without recurrence. This mechanism holds immense promise for precise and targeted drug delivery.

Moreover, VNB/PDA/Fe₃O₄ NPs exhibit noteworthy potential in photothermal therapy, magnetic targeting, MRI imaging, and chemotherapy. This versatile approach represents a significant advancement in cancer treatment modalities, offering a promising avenue for future research and clinical applications. Our work provides a nanomaterial endowed with dual-targeting capabilities for the synergistic treatment of cancer via photothermal and chemotherapy, demonstrating excellent application prospects in the future.

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Data Availability Statement
The data that supports the findings of this study is available from the corresponding author upon reasonable request.

References


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