Novel bacteriophage-based nanoligands

Marta Sofia Navalho Patinha

Abstract

Bacteriophages are viruses that are able to target and infect bacteria, having great specificity to these organisms. Due to a wide range of great properties – high specificity, great robustness, resistance to extremely harsh conditions – there's been an increasing interest in the use of bacteriophages in biorecognition events. On the other hand, there's also been great interest in the conjugation of biological molecules capable of targeting bacteria with nanoparticles. The main objective of this project is the development of a novel bacteriophage-based nanoligand, where magnetic nanoparticles are incorporated inside the phage capsid, allowing to overcome standard labelling disadvantages. As conjugation methods of phage with the MNPs both osmotic shock and sonication strategies were applied to T4 phages. Afterwards, the magnetic-phages are to be used in the development of magnetic-based detection assays. Phage-based magnetoresistive biosensors are already under development at INESC-MN and being explored for bacterial cells detection. In the future, the magnetic phages here developed will be tested, validated and incorporated as labelling entities in such magnetic biosensors. Future perspectives lie in the optimization of the conjugation processes, as well as investigation of new approaches, the following characterization of the magnetic phages in TEM and further testing in magnetic biosensors developed in INESC-MN.

Keywords: Bacteriophages, Bacterial Detection Methods, Nanoparticles, Magnetic Nanoparticles, Magnetophage, Novel Nanoligands

Introduction

The emerging resistance to antibiotics and the low rate of new antibiotic discovery turned pathogenic bacteria into a huge threat to human health, being a leading a cause of morbidity and mortality worldwide, causing millions of deaths and hospitalizations per year and generating a significant high social and economic impact worldwide. The most common sources of these infections are clinical, foodborne, airborne, and/or waterborne, where contaminations originated from these sources represent eternal challenges worldwide in the healthcare systems and food safety and environmental monitoring[1–3]. This led to the need of search for not only new methods of treatment but also for fast and reliable detection and identification methods of bacteria. Nowadays, conventional methods used for bacteria detection are dependent on the culturing and isolation of the target bacteria followed by biochemical confirmation. Although quite cheap and straight-forward, the conventional procedure is very time-consuming, having repercussions not only in healthcare but also in industry and security. Therefore, new detection methods are being introduced in the last few years. Among them are nucleic-acid-based (includes PCR and DNA micro-arrays), immune-based methods (includes ELISA and lateral flow immunoassays) and even mass spectrometry[4–6]. However, all of them have problems associated, such as the need for specialized equipment, for trained users and the expensive cost. So, there's a great need for the development of cheap, fast, specific, and sensitive diagnostic approaches for the detection of pathogenic bacteria detection. In response to this problem, there's been a rising interest in bioligands in biomolecular recognition events. Bacteriophages are viruses that only infect and replicate in bacteria. These viruses are one of the most the simple and most abundant organisms on Earth and are thought to exist in every ecosystem with an estimated number of 10^{31} phage particles in the biosphere, having a role in the continuous regulation of microbial ecology[7–9]. They pose great advantages, such as great specificity, robustness, resistance to harsh conditions and cheap preparation, making them a great candidate not only for phage-therapy but also as biorecognition elements. However, their potential can be empowered through the conjugation with other components. Magnetic nanoparticles have been attracting much interest as a labelling material for advanced biological

and medical applications, such as biomagnetic concentration and separation, drug delivery, magnetic resonance imaging, and hyperthermia. Presently, the most common way of conjugation of magnetic nanoparticles and biomolecules is through cross-linking or affinity molecules. However, these conjugation methods can negatively affect biorecognition events dependent on surface receptors. In this research project, the creation of a novel bacteriophage-based nanoligand through the filling of the phage capsid with magnetic nanoparticles will be evaluated.

Materials and Methods

1. Bacterial strains, bacteriophages and culture media

The bacteria and bacteriophages used in this study were: *Escherichia coli* (*E. coli*) with the corresponding bacteriophages, T4. *E. coli* bacteria and bacteriophages used were obtained from the company Dsmz (Braunschweig, Germany). The bacteria strains used in this study were grown on Tryptio-Casein Soy Broth (TSB) media at a temperature of 30-40ºC under agitation (200-300 rpm) or in solid plates containing Trypto-Casein Soy Agar (TSA). Both growth media were provided by Biokar Diagnostics (Beauvais, France).

2. Phage conjugation with magnetic nanoparticles:

2.1. Osmotic Shock:

Osmotic shock was performed as a method of conjugation for the interiorization of the permanent magnetic nanocomposite of magnetite (MNPs) into the bacteriophage's protein capsid. With this objective in mind, to study the efficiency of the method, three different conditions were tested: samples with bacteriophage only, and samples with bacteriophage with MNPs added after and before performing the osmotic shock. To the samples with MNPs, it was added approximately 10 mg of NdFeB magnetic particles (Magnequench), with an average diameter of 5 nm. Osmotic shock was performed using $C_2H_3NaO_2$ 4M as hypertonic solution and used in a proportion of 1:1, where 500 µL of phage solution was added to a sterile 50mL falcon, along with 500 µL of hypertonic solution. The samples were left incubating on ice, where the time of incubation was varied (5, 10, 15, 20 and 30 minutes). After the time of incubation, 50 mL of autoclaved chilled water was added. The obtained samples were characterized and stored at 4ºC. After the treatment, the samples were submitted to magnetic separation. Due to the large size of the falcons, an individual magnet was used as an option instead of a dedicated magnetic concentrator. The samples were submitted to 2 minutes of magnetic separation, with the magnet placed against the falcon wall, at the bottom. The supernatant was collected, and the magnetic pellet was washed with 5 mL of SM buffer. The samples were again magnetically separated for 2 minutes, and the supernatant collected. The same procedures were repeated one additional time, obtaining a magnetic pellet at the end. All samples – supernatant, washes supernatant and magnetic pellet - were collected, stored at 4ºC until further characterized. Further into testing, the final obtained magnetic pellet was washed with 5 mL of SM buffer two additional times, according to the last described method, resulting in a magnetic pellet. All samples – washes and magnetic pellet - were collected, stored at 4ºC till further characterized.

2.2. Sonication:

Sonication in ultrasound bath was performed as a second method of conjugation for the study of the interiorization of the MNPs into the bacteriophage's protein capsid. The sonication treatment was applied to samples of 1 mL of phage solution and to samples of 1 mL of phage solution with 20 mg of NdFeB magnetic particles. Sonication was performed in an Ultrasonic bath USC TH (VWR, Amadora, Portugal) at 45 kHz with an initial temperature of 10ºC. Five different sonication times were tested for each sample (15, 20, 30, 40 and 60 minutes). The obtained samples were characterized and stored at 4ºC till further testing. Afterwards, the samples were submitted to magnetic separation. For

that purpose, a DynaMag™ Magnet (Invitrogen, ThermoFisher) for 2 mL samples was used. The samples were submitted to 2 minutes of magnetic separation. The supernatant was collected, and the magnetic pellet was washed with 1 mL of SM buffer. The samples were again magnetically separated for 2 minutes, and the supernatant collected. The same procedures were repeated three more times, resulting in a total of four washes and one final magnetic pellet. The supernatants and the magnetic pellet were collected. All samples were stored at 4ºC till further testing.

3. Phage Characterization:

3.1. Phage Titration:

Phage titration was used as a characterization method for the study of the conjugation of MNPs with the bacteriophages in solution. To start the procedure, a pre-inoculum of the host-bacteria correspondent to the phage be amplified was prepared in a 15 mL falcon containing 5 mL of TSB medium and grown at 30-40ºC with vigorous agitation (20-300 rpm) overnight. After approximately 18h, the overnight grown cells were re-inoculated in about 5 mL of medium at an initial optical density at 600 nm (OD_{600}) of 0.1 as measured in the Hitachi U-2000 spectrophotometer. The cells were grown at 30-40ºC with vigorous agitation (20-300 rpm) till an OD_{600} of 0.25-0.30. Meanwhile, 100 µL of phage stock was serially diluted in 900 µL of SM buffer. After reaching an OD_{600} of 0.25-0.30, 20-100 µL $MgCl_2$ (Sigma-Aldrich, St.Louis, MO, USA) was added to the inoculum. The 100 µL of each appropriate dilution was added to 100-300 µL of $MgCl_2$ containing inoculum and incubated for 15-30 minutes at 30-40ºC without agitation. Then, to each dilution it was added 2-4 mL of molten top agar (3.5 g agar-agar, 12.5g TSB powder, 500 mL Milli-Q H_2O) with $MgCl_2$ (10 µL $MgCl_2$ per 1 mL of top agar), gently mixing it and pouring into TSA plates. The plates were incubated at 37ºC without agitation overnight. The titer of the samples was calculated.

3.2. Bradford Protein Assay:

Bradford assay was also used as a characterization method for the study of the conjugation of MNPs with the bacteriophages in solution. A microwell plate was used, where 50 µL of each sample and the controls were pipetted into the wells. The BSA samples were also pipetted into the wells in the same volume. Duplicates of all samples were made. Using a multichannel pipette, 200 µL of Coomassie solution were added to each well. The plate was mixed for 30 seconds and incubated for 10 minutes at room temperature. The absorbances where measured in a Multiskan FC Microplate Photometer (Thermo Fisher) at 595 nm. The samples protein concentration was obtained through the construction of a BSA standard calibration curve.

3.3. Transmission Electron Microscopy (TEM):

TEM was utilized with the finality of characterizing the bacteriophages and confirming if the conjugation of the particles with the phages was successful. The procedure was accomplished in MicroLab, IST (Lisbon, Portugal). Uranyless was used as a negative staining agent. The T4 phage solution and a sample with T4 phage solution with added MNPs in suspension were one time diluted for visualization, for the same reasons mentioned previously. Treated samples were used directly. The phage suspensions were dripped onto a carbon-formvar coated grid and fixed for 1 min. The liquid in excess was removed by tight contact with absorbent paper. The grid was left air-drying for 30 s at room temperature, followed by the negatively staining with UranyLess (Delta Microscopie, Mauressac, France).

Results and Discussion

1. *E. coli* phage osmotic shock treatment:

With the finality of characterizing the samples submitted to osmotic shock, a phage titration was performed according to the previously mentioned protocols. As

controls it was used a sample containing 500 µL of T4 bacteriophage diluted in 50 mL of autoclaved water and 500 µL of T4 bacteriophage with 10 mg of the magnetic nanocomposite MNPs (provided by INESC-MN) diluted in 50 mL of autoclaved water. The phage titration method is based on the counting of plaque forming units in a bacterial lawn, assuming that each plaque unit comes from one single phage that successfully infected the bacteria present in that region. However, the plaque counts on each plate should be between 30-300 to ensure the reliability of the method. With this in mind, after incubation, plaque units were counted to obtain the number of plaque forming units per milliliter (PFU/mL) using equation 1:

$$\frac{PFU}{mL} = \frac{Number\ of\ plaques\ counted\ x\ Dilution\ factor}{Inoculum\ Volume} \quad (1)$$

The results are shown in Figure 1 in PFU units.

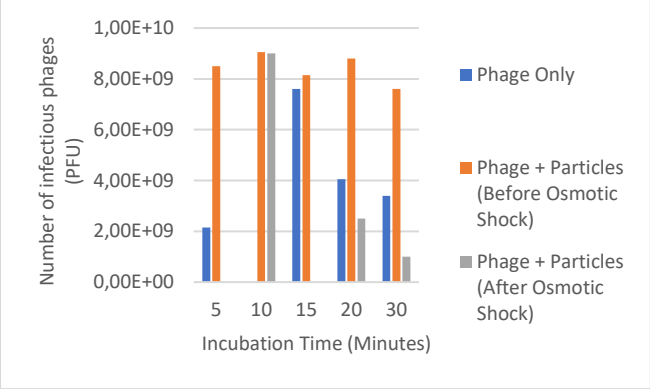

Figure 1 - Evolution of the number of T4 phages present in each sample after the osmotic treatment with the incubation time. Values from the condition Phage + Particles (After Osmotic Shock) at 5 minutes of incubation and Phage Only at 10 minutes of incubation were excluded.

The previously mentioned controls, the sample with only phage diluted and the sample with phage and MNPs diluted, were also titrated, resulting in a concentration of 2.86×10^8 PFU/mL for both samples. The number of infectious phages was also calculated for the controls, obtaining a value of 1.43×10^{10} for each control. When comparing the number of infectious phages present in samples treated with osmotic shock with the obtained in the control, an average infectivity reduction of 70% occurred. However, it is worth noting the obtained infectious phage number for 10 minutes of incubation time, where it appears that the phages did not lost infectious capacity. Additionally, it appears to have a slight increase in the number of infectious phages when compared to the control. Due to this pattern not being seen in any other sample, this result can be attributed to titration errors, not being accounted for the calculation of the average infectivity reduction. On a similar note, the samples correspondent to the addition of the MNPs after the submission of the phage particles to the osmotic shock conditions suffered an average infectivity reduction of 71% when compared to the control. However, is worth noting the obtained infectious phage number for 5 minutes of incubation time, where it appears to increase this number when compared to the control. The most probable cause for this obtained result is the occurrence of errors during the titration procedure, since this pattern was not found in any more samples. Additionally, when analysing the result obtained from the sample Phage + Particles (After) incubated for 15 minutes, it appears that it lost all infection capacity. However, when compared to the higher incubation times for the same sample, it still presents infectivity. Also, as seen in later chapters for the analysis of further testing, it appears to have infectious phages present in solution. So, the obtained result can also be attributed to titration errors and it was not accounted for the loss of infectivity. Regarding the incubation time, a similar result can be seen in both conditions (phage only and phage with particles were added after the treatment), where a gradual decrease in the number of infectious phages can be seen between 10, 20 and 30 minutes of incubation time, going to a loss of infectivity as high as 93% at an incubation time with the salt of 30 minutes for the condition Phage + Particles (After). To confirm this gradual decrease in infectivity, the titration of samples from incubation times 5 and 15 minutes should be repeated. Additionally, after some search in literature, it was found an article by Anderson *et. al* where it was reported that when submitting suspensions of T2, T4 and T6 phages to high concentrations of salt and then quickly diluted, the phages lost infectivity capacity and the presence of "ghost" phages in solution. So, is possible to assume that the decrease in the number of infectious phages

observed in the samples with only phage in solution can be due to either escape of the phage DNA from the capsid, consequently producing ghost phages in solution, or to the complete busting of the phage capsid. However, when observing the samples with osmotically treated phages with MNPs added posteriorly, it was obtained the highest loss of infectivity when incubating with 30 minutes of hypertonic solution. It's possible that the same scenario happened, since the average loss of infection value is very close to the obtained in the samples with only phage. However, when observing the results for the phages treated along with the MNPs, it was obtained a smaller percentage of loss of infectivity (41%). One possible scenario is that, when submitting the phages to osmotic shock, the capsid was disrupted enough to allow the entrance of MNPs. However, these particles could have avoided somehow the release of the phage DNA from the capsid, and the phages maintained their infection capacity. A Bradford Protein Assay was also performed in the same samples, however it appeared that the MNPs reacted with the Coomassie reagent and, consequently, no conclusions could be made. To verify if the phages where magnetized, meaning conjugated with the added MNPs, the samples with MNPs added to the phage solution before and after performing the osmotic treatments were submitted do magnetic separation. In a first instance, only two washings were performed, resulting in a magnetic pellet, and all the samples were collected and stored at 4ºC (supernatant, 1st wash, 2nd wash and magnetic pellet). The obtained magnetic pellet was then washed two more times and all the samples were also collected and stored at 4ºC (3rd wash, 4th wash and magnetic pellet). As a way of characterizing the magnetic samples, all collected aliquots were titrated (Results Not Shown). It was possible to observe that samples with phage and MNPs added before performing the osmotic shock had a decrease of more than 90% of the number of infectious phages from the sample before the magnetic separation to the pellet, where more than 90% of the phages stayed in the supernatant. Regarding the results for the magnetic pellet obtained at the end of the first two washes, it is visible a slight increase in the number of infectious phages along the incubation times. When comparing the results from the first obtained pellet to the second, it should be expected a decrease in the number of phages with the number of washes, as observed in the control. However, when comparing both pellets, it was possible to see that in almost all incubation times, the number of infectious phages is maintained. As for the results for samples corresponding to the condition of the addition of MNPs, similar results are observed. When comparing the results from the pellet to the number of infectious phages that where in the sample before the magnetic separation, it is possible to see a reduction of more than 90% of the number of infectious phages in the magnetic pellet, where the majority of phages remained in the supernatant. Regarding the results for the magnetic pellet obtained at the end of the first two washes, the number of infectious phages is maintained along the incubation times. When comparing the results from the first obtained pellet to the second, it is possible to observe an average loss of 99,44% of infectivity from the first pellet to the second, which is consistent with the results observed in the control. This could lead to believe that when submitting the phages and the MNPs to osmotic shock, simultaneously, it could lead to the magnetization of the T4 phages, which could explain the maintenance of the number of infectious phages with the washes. However, the same treatment with the MNPs added after could not promote the magnetization of the phages, leading to the loss of infectious phages along washing steps. It is also worth mentioning that, in case of magnetization, only a small portion of phages was conjugated. Remaining in that hypothesis, this small number of magnetized phages could be due to the mass of MNPs added is not enough for the number of phages in solution. To validate this hypothesis, the samples corresponding to 20 and 30 minutes of incubation with MNPs added before the osmotic shock were chosen for visualization, as well as dilluted T4 phage solution and T4 phage solution with added MNPs in suspension for the native phage and MNPs

characterization. As mentioned previously, Uranyless was chosen as negative staining agent. However, it was not possible to obtain images for the native T4 phage and the MNPs. A probable cause for this occurrence is the dilution being too high to capture the phage and MNPs in the grid. In further TEM visualizations, the best option is to use non-diluted samples. The images from the treated samples are shown in Figure 2:

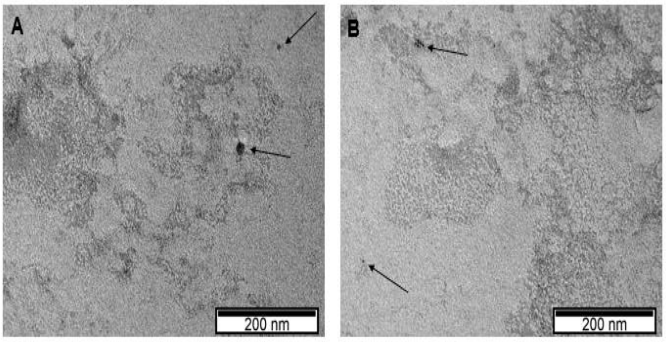

Figure 2 - Obtained TEM visualizations with Uranyless used as negative staining agent. Images **(A)** and **(B)** correspond to the sample with T4 bacteriophage with MNPs added before osmotic shock incubated for 20 minutes. The capture of MNPs agglomerates is marked with arrows.

Observing Figure 2, T4 bacteriophages images were not captured, not being possible to verify the location of MNPs on the inside of the phage capsid. However, as identified in both figures, agglomerates of MNPs are shown. The particles appear to have around 4 nm of diameter, confirming to be much smaller than the T4 phage capsid, reported in literature to be 120 nm long and 80 nm wide, as mentioned previously. It is possible that, due to the performance of osmotic shock and the magnetic separation, the phages were too diluted for visualization. In the future, the samples should be concentrated before visualization to try to overcome this problem. However, some type of reaction appears to have taken place, possibly affecting the visualization of the samples.

2. *E. coli* phage sonication treatment:

Sonication is a physical method of disruption used in a variety of biological entities. In an article published by Machida *et. al*, it was used sonication as a capsid disruption method for the liberation of a coliphage-associated sialidase[12]. So, sonication was proposed as a method for the conjugation of the magnetite MNPs inside the T4 phage capsid. A bath sonication was first proposed since the sonication conditions would be less harsh than the direct sonication provided by a probe sonicator, having less probability of completely disrupting the capsid and only permeabilizing it enough to allow the internalization of the particles. The protocol described in section 2.2 of Materials and Methods was executed and all the samples were titrated. As controls, it was used a sample containing 1 mL of T4 bacteriophage with 20mg of magnetite MNPs, submitted to the same protocol of magnetic separation, the samples with 1mL of phage solution sonicated at each exposition time. When comparing the values of the samples with phage with the values obtained for the solution with phage and MNPs before being submitted to sonication to their initial phage concentration – $2{,}86 \times 10^{10}$ PFU/mL – it was observed that no lytic activity was lost, meaning that the phages integrity was maintained. In addition, is possible to see an increase in the phage concentration with the sonication exposure time. A possible justification for these results is the decrease on the number of phage aggregates in solution. In an experiment reported by Machida *et. al*, after the sonication of MS2 phage suspension, an increase in the phage number in solution was reported[12]. The authors associated the increase to the reduction of the small phage clusters present in solution. The same increase in the phage concentration can be observed in the supernatant and among the four performed washes, resulting in an increase of up to one magnitude value with the sonication exposure time. When treating the obtained titration results from the pellet, the concentrations from incubation times 15, 20, 40 and 60 minutes where not possible to calculate due to the obtention of plaque counting out of the 30-300 range necessary for the reliability of the method. However, when observing the plaques from 30, 40 and 60 minutes of exposure time, it was possible to see a gradual increase in the number of plaque forming units. This gradual increase in the phage concentration with the exposure time can be justified with the decrease of phage agglomerates in solution, leading to a higher

number of phages in the pellet. However, this increase can also be due to the possible magnetization of the phages. However, such magnetization can be due to the internalization of the MNPs or to the adsorption of the particles to the exterior of the phage capsid. This hypothesis can only be confirmed when submitting the pellet samples to TEM image capture.

Conclusion and Future Work

Bacteriophages have available a wide range of interesting properties that are desirable for the detection of pathogenic bacteria. On the other hand, magnetic nanoparticles present unique properties that, conjugated with bacteriophages, could unravel novel properties for the application of bacterial detection. The main objective of this project is the development and optimization of a conjugation process to obtain magnetic phages. Since the objective of this thesis is the internalization of the MNPs into the bacteriophage capsid, methods that could possibly permeabilize the capsid were chosen. Both results from the osmotic shock and bath sonication where not conclusive in regard to the efficiency of the processes in the conjugation of the particles. The characterization tests should be repeated, with the optimization of the uranyless staining protocol for TEM visualization. However, in the future, some other conjugation methods and conditions would be applied. Since in the chemical treatment used did not totally diminished the lytic activity of the phages, is probable that the conditions used were not enough to completely destabilize the protein capsid of the T4 bacteriophages, but it could be enough to lead to the creation of gaps that would allow the release of DNA and possibly the internalization of bacteriophages. Additionally, Anderson *et. al* reported that when submitting suspensions of T2, T4 and T6 (large virus) phages to high concentrations of salt and then quickly diluted, the phages lost infectivity capacity and the presence of “ghost” phages in solution. However, interestingly, the authors also reported that when submitting suspensions of T1, T3, T5 and T7 (small virus) to the same conditions, they kept their capacity of infection and appeared to remain intact[10]. Jurczak-Kurek *et al.*, as a method to study the morphological and biological properties of a wide group of bacteriophages isolated from urban sewage, proceeded to submit the phages to osmotic shock conditions (same conditions as the protocol used in the article by Anderson *et al.*). The authors reported that from 83 phages tested, 21 where susceptible to osmotic shock, where 18 of them presented larger capsid size compared to the rest and belonging to the *Myoviridae*[11]. T4 is described in literature as having a 120 nm long and 86 nm wide icosahedral capsid[13], confirming to be much larger than odd-numbered phages, like a T7 phage, which is described as a much smaller phage, having a capsid around 60 nm wide[14]. So, in the future, it should also be taken into account the size of the bacteriophages, since it appears that larger bacteriophages appear to be more susceptible to osmotic shock conditions than smaller phages. With this in mind, different phages with different sizes should be submitted to the same treatments. Also, higher salt concentrations (e.g. 5M and 6M) should also be tested. Another chemical method could also be tested as conjugation method, such as alkaline treatment. Liu *et. al*. reported the obtention of “ghost” phages when submitting them to an alkaline treatment, proving to destabilize the protein capsid to remove the phage DNA[15]. However, the applied conditions are not reported by the authors. On the other hand, Muller-Salamin *et. al*. reported the obtention of gaps or openings in T4 capsid when submitting them to a treatment of pH 11.0 in 7M of urea[16]. These small gaps could allow the entrance of the MNPs, magnetizing the phages. Even though this protocol was only tested in capsids and not the whole phage, the maintaining of the tail components would need to also be evaluated. Regarding the results from bath sonication, they were not conclusive, especially since TEM visualization was also not possible. So the repetition of the test would be needed. However, different temperatures could be tested to promote the capsid proteins denaturation, which could increase the probability of the internalization of the MNPs. Probe sonication would

also be tested and compared to the results obtained for bath sonication, since the application of the ultrasounds it is directly applied into the sample, leading to a bigger disruption of the capsid and possibly causing more movement of the magnetic particles and "pushing" the MNPs into the interior of the capsid. The efficiency of the techniques and characterization of the bacteriophages will be evaluated through TEM with the new staining agent. To further evaluate the validation and detection of the magnetic phages, magnetoresistive sensors developed by INESC-MN would be used.

Acknowledgements: This document was written and made publicly available as an institutional academic requirement and as a part of the evaluation of the MSc thesis in Biotechnology of the author at Instituto Superior Técnico. The work described herein, was performed at the institute for Bioengineering and Biosciences (iBB) at Instituto Superior Técnico (Lisbon, Portugal), during the period of September 2019 to November 2020, under the supervision of Dr. Verónica Romão and Prof. Ana Azevedo.

References

1. Farooq, U., Wajid Ullah, M., Yang, Q. & Wang, S. Applications of Phage-Based Biosensors in the Diagnosis of Infectious Diseases, Food Safety, and Environmental Monitoring. in *Biosensors for Environmental Monitoring* (IntechOpen, 2019). doi:10.5772/intechopen.88644
2. Ahmed, A., Rushworth, J. V., Hirst, N. A. & Millner, P. A. Biosensors for whole-cell bacterial detection. *Clin. Microbiol. Rev.* **27**, 631–646 (2014).
3. Farooq, U., Yang, Q., Wajid Ullah, M. & Wang, S. Principle and Development of Phage-Based Biosensors. in *Biosensors for Environmental Monitoring* (IntechOpen, 2019). doi:10.5772/intechopen.86419
4. Farooq, U., Yang, Q., Ullah, M. W. & Wang, S. Bacterial biosensing: Recent advances in phage-based bioassays and biosensors. *Biosensors and Bioelectronics* **118**, 204–216 (2018).
5. Richter, Ł., Janczuk-Richter, M., Niedziółka-Jönsson, J., Paczesny, J. & Hołyst, R. Recent advances in bacteriophage-based methods for bacteria detection. *Drug Discovery Today* **23**, 448–455 (2018).
6. Maugeri, G., Lychko, I., Sobral, R. & Roque, A. C. A. Identification and Antibiotic-Susceptibility Profiling of Infectious Bacterial Agents: A Review of Current and Future Trends. *Biotechnol. J.* **14**, 1700750 (2019).
7. Sharma, S. *et al.* Bacteriophages and its applications: an overview. *Folia Microbiol. (Praha).* **62**, 17–55 (2017).
8. Casey, A., Coffey, A. & McAuliffe, O. Genetics and Genomics of Bacteriophages. in *Bacteriophages* 1–26 (Springer International Publishing, 2017). doi:10.1007/978-3-319-40598-8_5-1
9. Hungaro, H. M., Lopez, M. E. S., Albino, L. A. A. & Mendonça, R. C. S. Bacteriophage: The Viruses Infecting Bacteria and Their Multiple Applications. in *Reference Module in Earth Systems and Environmental Sciences* (Elsevier, 2014). doi:10.1016/b978-0-12-409548-9.09039-4
10. Anderson, T. F. The reactions of bacterial viruses with their host cells. *Bot. Rev.* **15**, 464–505 (1949).
11. Jurczak-Kurek, A. *et al.* Biodiversity of bacteriophages: Morphological and biological properties of a large group of phages isolated from urban sewage. *Sci. Rep.* **6**, 1–17 (2016).
12. Machida, Y. *et al.* Structure and function of a novel coliphage-associated sialidase. *FEMS Microbiol. Lett.* **182**, 333–337 (2000).
13. Rao, V. B. & Black, L. W. Structure and assembly of bacteriophage T4 head. *Virology Journal* **7**, 356 (2010).
14. Deng, X., Wang, L., You, X., Dai, P. & Zeng, Y. Advances in the T7 phage display system (Review). *Molecular Medicine Reports* **17**, 714–720 (2018).
15. Liu, C. *et al.* Magnetic viruses via nano-capsid templates. *J. Magn. Magn. Mater.* **302**, 47–51 (2006).
16. Muller-Salamin, L., Onorato, L. & Showe, M. K. *Localization of Minor Protein Components of the Head of Bacteriophage T4*. *JOURNAL OF VIROLOGY* (1977).