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Development of wound dressings for biofilm inhibition by means of inkjet printing

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Abstract

Printing technology has given new opportunities for the fabrication of pharmaceutical dosage forms. The utilization of inkjet printing allows obtaining drug delivery systems with controlled and precise dosing of low dose medications. The healing process of wounds is hindered due to the biofilm formation by the bacteria. Therefore, effective antimicrobial treatments are crucial in wound care.

In this study, the use of inkjet printing technology for the fabrication of antibacterial drug formulations for topical applications was investigated. The customized formulations with antibiotic gentamicin sulfate printed on medical grade silicon sheeting were prepared. The results showed that the inhibition of biofilm formation was achieved with all the printed formulations in pre-exposure assay to Staphylococcus aureus on static method agar plates.

This study provided insight into the feasibility of inkjet printing for the fabrication of topical drug delivery systems. By adjusting the dose and drug-covered area in the wound dressings, inkjet printing could provide the flexibility that is needed to improve the personalization of wound care.

Introduction

In recent decades, the research focusing on the application of inkjet printing for the fabrication of pharmaceuticals has increased [1]. Printing could be feasible in producing dosage forms that are tailored according to the individual needs of the patient [2]. Compared to the fabrication of conventional solid dosage forms this approach as many advantages including the decreased number of manufacturing steps and the alternatives for designing formulations with versatile dosage, drug release profile and drug combinations [3]. The flexibility of this method lies in the fact that the properties of the dosage form can be tailored by modifying the parts of the formulations (e.g. substrate, ink, coatings) and/or the process parameters (e.g. printing pattern, droplet volume, drying time) separately. Inkjet printing is suitable for depositing precise doses of low-dose medications on a pre-defined location on a substrate [2,3,4]. The drug compounds can be dissolved or dispersed in a suitable inkbase solution.

Previously, the printed formulations have been designed mainly for oral administration [2,5]. For that application, both thermal and piezoelectric inkjet printing methods have been used to apply a well-distributed layer of drug-loaded ink onto the surface of a substrate [2]. The flexibility of the dosing can be achieved by using inks with different drug concentrations [6], varying the printed area [7], resolution [7] or the number of printed layers [8]. Furthermore, the selection of substrates is important, since, for example, porous substrates have been suggested to be beneficial for enhancing the stability of the solid state of the drug [4].

Biofilms are structured communities of bacterial cells that are firmly surrounded by a matrix of extracellular polymeric substance (EPS) that acts as a protection layer against biocides, antibiotics and host immune response and as an adhesion mechanism [9,10]. The infectious biofilms are formed from planktonic bacteria on the surfaces of biomedical devices or in human tissue [10]. The antibiotic therapies are generally inefficient in eliminating bacteria in mature biofilms, therefore, strategies for preventing biofilm formation and obtaining a bactericidal effect in the biofilm growth state are preferred [10].

There are several types of wounds and a variety of wound dressings with different properties [11]. Especially chronic wounds are a widely recognized health problem [10]. The risk for the development of chronic wounds is increased for patients with diabetes and cardiovascular diseases [10]. Microbial control in wound care is crucial during the wound healing process, because of the biofilm formation by the bacteria that interferes with the healing process [12]. A study by James et al. (2008) showed that the presents of biofilms in chronic wounds was 10 times higher compared to acute wounds [13]. The predominant species identified in these wounds were *Staphylococcus, Pseudomonas* and *Enterococcus* [13]. Thus, treatments that are effective against biofilm formation are greatly needed to reduce the occurrence of chronic wounds [12].

Wound dressings are divided into several categories depending on their use (occlusive, absorbent, adherence), material (polyurethane, alginate, collagen, silicone) or physical form (film, foam, gel) [14]. These dressings are required to support one or more aspects of the wound healing process [11,14]. Local drug delivery increases the therapeutic effect at the wound site and also decreases the risk for systemic side effects [11]. However, the disadvantages include an increased risk for systemic exposure when applied on large areas, dosing challenges, potential skin reactions and interference with the wound healing process [15]. Nevertheless, various therapeutically active agents can be used to promote wound healing – topical disinfectants (e.g. ethanol), antiseptics (e.g. hydrogen peroxide, chlorhexidine) and bioactive components (e.g. antibiotics, anti-inflammatory drugs) [10]. By incorporating an active pharmaceutical ingredient, for instance an antibiotic, into/onto a wound dressing, infections can be fought or prevented by inhibiting the biofilm formation in the damaged tissue [10,11].

In this study, the exploitation of inkjet printing in the preparation of topical formulations with antimicrobial agents was investigated [16]. Model formulations were prepared by printing an antibiotic gentamicin sulfate onto a medical grade substrate. The effect of the printed drug delivery systems on the biofilm inhibition was evaluated by a static biofilm method. According to our knowledge there are no previous reports on the use of inkjet printing for the manufacturing of

wound dressings. Due to its flexibility this approach could improve the personalization of wound care by adjusting the dose and drug-covered area in the wound dressings.

Materials and Methods

Materials

A broad spectrum antibiotic, gentamicin sulfate (Sigma-Aldrich, China) was used as model drug compound (Figure 1). Propylene glycol (PG) (99.5%, SAFC, Germany) and purified water (Milli-Q) were used to prepare the ink formulations. A non-sterile medical grade silicone sheeting (BioPlexus, USA) and standard copier transparency film (Folex, Switzerland) were used as substrates.

Inkjet Printing

Inks with two different drug concentrations, 3 µg/µl and 12 µg/µl, were prepared by dissolving gentamicin sulfate in the inkbase consisting of a mixture of PG and water (50:50 vol%).

A CAM 200 contact angle goniometer (KSV Instruments Ltd, Finland) was used for surface tension measurements and the rheological properties of the inkbase were characterized using a Paar Physica MCR 300 modular compact rheometer (Anton Paar GmbH, Germany) with a double-gap concentric cylinder geometry.

A PixDro LP 50 (Roth & Rau, the Netherlands) piezoelectric inkjet printer with a Spectra SL printhead (128 nozzles, Ø 50 µm) was used for printing. The ink formulations were filtered through sterile 0.2 µm cellulose acetate syringe filters (Whatman, Germany) prior to printing. The printing was performed at ambient conditions (temperature of 20-21 °C, relative humidity of 20-25%). One nozzle was used for the ink deposition at a resolution of 500 dpi according to the tailored patterns (Table 1). For further analysis, the printed samples were cut out into square-shaped samples of approx. 1 cm × 1 cm regardless of the pattern.

Table 1. The design of the printed formulations.

Pattern	■	+	⊡
Printed area	1 cm^2	0.2 cm^2	Manually pipetted dot
Ink concentration	3 µg/µl		12 µg/µl
	12 µg/µl		
Printed layers	1 layer	5 layers	-

Characterization of the printed formulations

Visual characterization of the printed formulations was performed by optical microscopy (Evos XL, AMG, USA).

The solid state properties of the printed gentamicin sulfate were evaluated by an attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy (Spectrum Two, PerkinElmer, UK) and a differential scanning calorimetry (Q2000 DSC, TA instruments, USA).

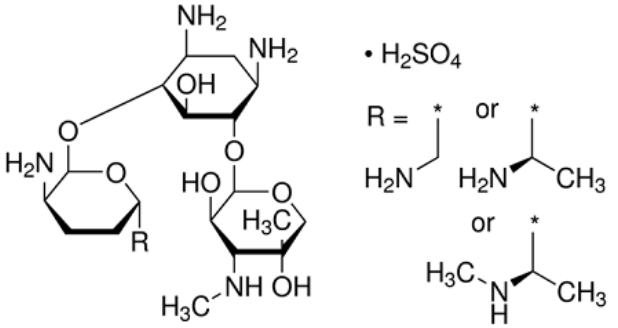

***Figure 1**. Molecular structure of gentamicin sulfate.*

Drug quantification

The gentamycin sulfate content in the printed formulations was determined by a colorimetric assay [17]. Ninhydrin powder (≥95%, Sigma-Aldrich, India), potassium phosphate monobasic powder (99.5-100.5%, Sigma-Aldrich, Germany) and sodium hydroxide pellets (Ph.Eur., Sigma-Aldrich, Germany) were used for preparing the reagent solutions. An ultraviolet-visible spectrophotometry (Lambda 25, PerkinElmer, USA) at 400 nm was used for drug detection. First, the samples were immersed in 1ml of pH 7.4 buffer solution and incubated at room temperature for 24 h. After that 900 µl of the sample solution was transferred into the reaction tubes and 270 µl of 1.25% ninhydrin solution was added (5:1.5 vol% ratio of gentamicin solution and ninhydrin solution). The reaction was conducted in a pre-heated oven at 95 °C for 30 min and stopped by placing the samples on an ice bath. The content of the printed formulations was determined in triplicate. The calibration was obtained in a gentamicin sulfate concentration range of 2.5-50 µg/µl in the same reaction conditions.

Biofilm inhibition studies of the printed formulations

The efficacy of the drug was determined against a methicillin-susceptible strain of Staphylococcus aureus (ATCC 25923). An adapted version of a previously described method was performed for culturing biofilms on tryptic soya agar plates [18]. The details of the pre-exposure assay with gentamicin sulfate against *S. aureus* on static biofilm method plates has been described by Öhman [16]. The dose-response curve was established by conducting a pre-exposure assay against S. aureus with different range of concentrations. The appropriate amount of gentamicin in the printed samples was determined based on the dose-response trials, in order to obtain a clear and steady biofilm inhibition.

The printed cross-pattern and full area samples were tested for their inhibitory effect on biofilm formation on static method agar plates. The biofilm was cultured by pipetting a bacterial suspension (1:10 vol% ratio of *S. aureus* and tryptic soy broth inoculum) onto a sterile filter paper (Whatman, Germany) placed on top of the agar plate. The printed samples and the control samples (borosilicate glass coupons with and without gentamicin) were placed on top of the inoculated filter paper with the treated or printed side facing a filter paper. The effect of printed pattern and dose on the inhibition level was determined by viable cell counts analysis of the printed samples after a 2 h or 24 h incubation period in a humidified incubator at 37 °C and 96% relative humidity. Three or four

biological replicates were analyzed per sample. Samples printed with the drug-free inkbase were used as controls.

GraphPad Prism v. 5.00 for Windows (GraphPad Software, U.S.A.) was used for the calculations and statistical analysis.

Results

Characterization of the printed formulations

The physical properties of the ink were characterized to predict the printability of the ink solution. The amount of the viscosity modifying agent PG in the inkbase solution was selected based on the rheological properties. The measured surface tension (44.0 mN/m), viscosity (6.53 mPa·s), and *Z* value (7.33) of a 50:50 vol% mixture of PG and water was shown to be most suitable for printing. The *Z* value was determined based on an approximate to the Navier-Stokes equation (1) as the inverse (*Z*) of the Ohnesorge number, where d is the nozzle diameter (m), ρ is the density (kg/m^3), γ is the surface tension (N/m) and η is the viscosity (Pa·s). The *Z* value within the range of $4 \leq Z \leq 14$ has been suggest to the be suitable for inkjet printing [19].

$$Z = (d\rho\gamma)^{1/2}/\eta \quad (1)$$

The microscopic imaging (Figure 2) demonstrated that the ink was distributed onto the substrate evenly with a single printed layer. Due to the limited absorption capacity and the hydrophobicity of the substrate, printing of several layers (cross-pattern samples) resulted in merged droplets and a higher variation in droplet sizes.

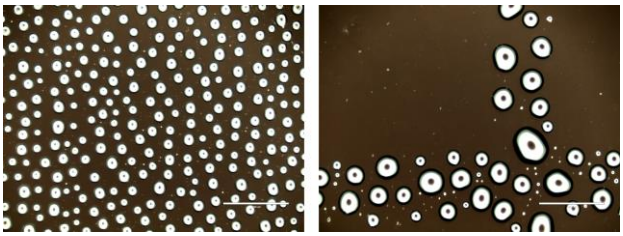

Figure 2*. Microscopic images at 4.0x magnification of gentamicin sulfate printed samples with a single layer (left) and 5 layers (right) on silicone. Bar = 1000 µm.*

Despite the low drug amount, characteristic absorption peaks at 1622 and 1525 cm^{-1} for gentamicin sulfate were detected for the printed samples on the ATR-FTIR spectra. Furthermore, the DSC analysis demonstrated the thermal stability of the gentamicin sulfate. In the printed samples, the DSC was able to detect the melting decomposition endotherm for gentamicin sulfate at 242.16 °C. The spectroscopic and thermal analysis strongly suggested that the properties of the drug were not altered by the printing process.

The printed drug amount was detectable in the formulations printing with a 12 µg/µl ink (Table 2). The printed samples on transparency film were used as reference because of the inert nature of the substrate. A day-to-day variation caused by the optimization of the printing settings was seen with the samples printed on silicone. Previously, Wickström et al. (2015) reported that the printed drug amount per layer decreased when a dose escalation by increasing the number of printed layer was applied, indicating that printing a low concentration ink in several layers could result in less accurate dosing [8]. Here, a very high variation in the cross-pattern samples (5 layers) was attributed mostly to the clogging problems and a consequent inconsistence in the ink flow during the printing task. It was suggested that an online monitoring of the droplet ejection could be beneficial for detecting clogging problems and evaluating the droplet size uniformity.

Table 2. The measured gentamicin sulfate content (µg) with relative standard deviation (RSD) in formulations printed with 12 µg/µl ink.

Substrate	Pattern	Content (µg)	RSD
Silicone	Cross-shaped	3.2 ± 2.9	89.5%
	Full area	10.8 ± 3.2	29.5%
Transparency film	Cross-shaped	12.3 ± 0.6	4.9%
	Full area	11.4 ± 0.5	4.4%

Antimicrobial properties of the printed formulations

The printed amount per formulations, and thus the used ink concentration, was determined beforehand based on the exhibited inhibition levels in the dose-response trials. The dose-response curve between the biofilm inhibition and the drug concentration is shown on Figure 3. The 50% of the maximal inhibitory concentration (IC50) of gentamicin was estimated at 256 µM (GraphPad Prism software). The aim was to prepare samples that would give a steady > 80% inhibition, thus, samples with approx. 10 µg were designed. The inhibition percentage of the coupons with 10 µg of gentamicin sulfate was 88.43 ± 7.92% (n=3) and the initial concentration (671 µM) corresponded to 2.62 times the estimated IC50 concentration.

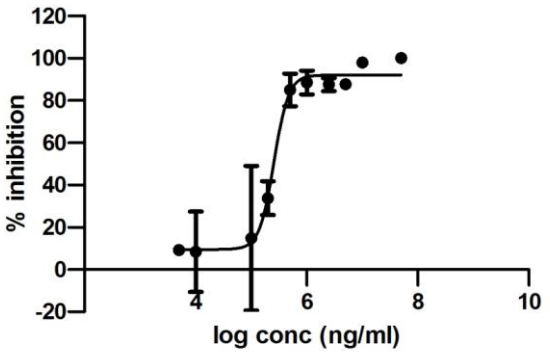

Figure 3. *Dose-response curve demonstrating the % inhibition of S. aureus on static biofilm method plates plotted against gentamicin sulfate concentration in Mueller-Hinton broth (mean ± SD, n=23).*

The effect of the printed dose and the pattern on the antimicrobial properties of the printed formulations was studied by determining the inhibition of *S. aureus* biofilm formation after a 2 h and 24 h incubation period. The effect of the dose on the biofilm inhibition can be seen in Figure 4. And Figure 5 demonstrates the effect of the printed pattern on the inhibition levels.

After a 2 h incubation period the lower dose samples showed an inhibition of 61.1 ± 7.3% (cross-pattern) and 69.6 ± 12.6% (full area). And the biofilm inhibition for the higher

dose samples was 95.1 ± 1.2% (cross-pattern), 93.2 ± 8.2% (full area) and 66.3 ± 14.5% (dot).

After 24 h of incubation the biofilm inhibition levels remained at 68.5 ± 15.0% (cross-pattern) and 71.5 ± 35.6% (full area) for the lower dose samples and at 87.3 ± 7.1% (cross-pattern), 87.9 ± 7.4% (full area) for the higher dose samples. A noticeable difference in the inhibition was seen for the manually pipetted dot samples, where the inhibition increased up to 83.6 ± 3.6% after 24h.

The results showed that the pattern of the printed formulations did not affect the biofilm inhibition at higher concentrations. It was suggested that the printed pattern/ drug-covered area might be of significance when low doses of antibiotics are used. In Figure 4, a slight difference, though statistically not significant, could be seen between the cross-shaped and full area samples at lower concentrations after 2 h of incubation.

As expected the biggest difference was seen between the manually prepared samples (dot) and the printed samples that contained the same amount of gentamicin sulfate (Figure 5). This was attributed to the significant contrast in the drug-covered area that is exposed to the bacteria and the diffusion rate of the drug in the static analysis settings.

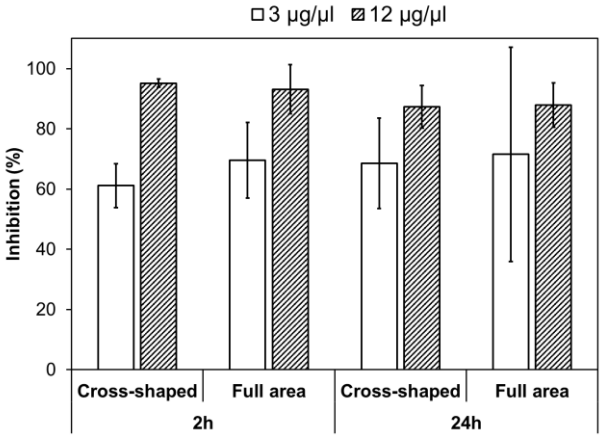

***Figure 4**. The reduction in viability counts of S. aureus bacteria after 2h and 24h incubation shown as inhibition % for formulations printed with different concentrations (n=3).*

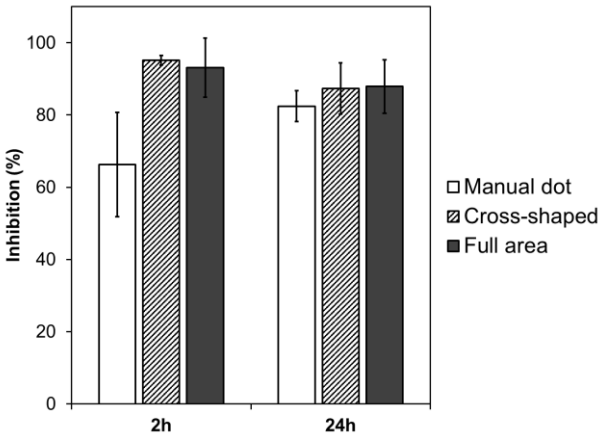

***Figure 5**. The reduction in viability counts of S. aureus bacteria after 2h and 24h incubation shown as inhibition % for formulations printed with different patterns (n=3).*

It should be noted that the high deviations in the results of the antimicrobial studies were caused by the variable drug content (Table 2). The effect of the API in the printed formulations remain unchanged after a short storage time (1 week) at 8 °C.

Gentamicin is a fast-acting antibiotic, reaching a maximum concentration after an intramuscular or intravenous dose after 30-60 min (T_{max}) and having a biological half-life ($T_{½}$) of 2-3 h. Therefore, enhancing the efficacy of gentamicin by combining with other antibacterial agents or prolonging the action by modified-release could be useful.

Conclusions

In this study, the model topical formulations with an antibiotic gentamicin sulfate were prepared by inkjet printing. The effect of the printing pattern on the biofilm inhibition was investigated.

The solid-state characterization of the printed formulations indicated that printing process did not alter the properties of the drug. The drug content analysis showed that the optimization of the printing process affected the day-to-day dosing precision. Furthermore, the printing of several layers of ink on top of each other limited the visual evaluation of the printing quality.

The printed formulations with approx. 10 µg per 1 cm^2 exhibited over 90% inhibition of biofilm formation already after 2 h in pre-exposure to *S. aureus* irrespective of the printed pattern. The rapid effect on the biofilm inhibition has shown to be characteristic for the drug. The results from the anti-biofilm studies suggested that the printing pattern might be of importance when low doses of antibiotics are used.

In conclusion, this study demonstrates the feasibility of inkjet printing as an alternative method for the preparation of topical formulations with variable drug content for personalized therapy. Thus, additional studies could be targeted at exploring the potential of inkjet printing for tailoring topical drug formulations for wound care purposes.

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References

[1] M. Alomari, F. H. Mohamed, A. W. Basit, S. Gaisford, Personalised dosing: Printing a dose of one's own medicine, Int. J. Pharm., 494(2), 568 (2015).

[2] R. Kolakovic, T. Viitala, P. Ihalainen, N. Genina, J. Peltonen, N. Sandler, Printing technologies in fabrication of drug delivery systems, Expert Opin. Drug Deliv., 10(12), 1711 (2013).

[3] N. Scoutaris, M. R. Alexander, P. R. Gellert, C. J. Roberts, Inkjet printing as a novel medicine formulation technique, J. Control. Release., 156, 179 (2011).

[4] N. Sandler, A. Määttänen, P. Ihalainen, L. Kronberg, A. Meierjohann, T. Viitala, J. Peltonen, Inkjet printing of drug substances and use of porous substrates-towards individualized dosing, J. Pharm. Sci., 100(8), 3386 (2011).

[5] M. Preis, J. Breitkreutz, N. Sandler, Perspective: Concepts of printing technologies for oral film formulations, Int. J. Pharm., 494, 578 (2015).

[6] A. B. M. Buanz, M. H. Saunders, A. W. Basit, S. Gaisford, Preparation of personalized-dose salbutamol sulphate oral films with thermal ink-jet printing, Pharm. Res., 28(10), 2386 (2011).

[7] N. Genina, D. Fors, M. Palo, J. Peltonen, N. Sandler, Behavior of printable formulations of loperamide and caffeine on different substrates – Effect of print density in inkjet printing, Int. J. Pharm., 453, 488 (2013).
[8] H. Wickström, M. Palo, K. Rijckaert, R. Kolakovic, J. O. Nyman, A. Määttänen, P. Ihalainen, J. Peltonen, N. Genina, T. de Beer, K. Löbmann, T. Rades, N. Sandler, Improvement of dissolution rate of indomethacin by inkjet printing, Eur. J. Pharm. Sci., 75, 91 (2015).
[9] J. W. Costerton, P.S. Stewart, E. P. Greenberg, Bacterial biofilms: a common cause of persistent infections, Science, 284, 1318 (1999).
[10] T. Bjarnsholt, O. Ciofu, S. Molin, M. Givskov, N. Høiby, Applying insights from biofilm biology to drug development – can a new approach be developed?, Nat. Rev. Drug Discov., 12(10), 791 (2013).
[11] J. S. Boateng, K.H. Matthews, H. N. E. Stevens, G. M. Eccleston, Wound healing dressings and drug delivery systems: a review, J. Pharm. Sci., 97(8), 2892 (2008).
[12] K. R. Kirker, P. R. Secor, G. A. James, P. Fleckman, J. E. Olerud, P. S. Stewart, Loss of viability and induction of apoptosis in human keratinocytes exposed to Staphylococcus aureus biofilms *in vitro*, Wound Repair Regen., 17(5), 690 (2009).
[13] G. A. James, E. Swogger, R. Wolcott, E. deLancey Pulcini, P. Secor, J. Sestrich, J. W. Costerton, P. S. Stewar, Biofilms in chronic wounds, Wound Repair Regen. 16(1), 37 (2008).
[14] G.M. Eccleston, Wound dressings in Aulton's pharmaceutics: the design and manufacture of medicines, 3rd edition (Churchill Livingstone, 2007), pp. 598-605.
[15] B. A. Lipsky, C. Hoey, Topical antimicrobial therapy for treating chronic wounds, Clin. Infect. Dis., 49(10), 1541 (2009).
[16] J. Öhman, Development of anti-biofilm wound dressings by inkjet printing, MSc thesis, Faculty of Science and Engineering, Åbo Akademi University, Finland (2016).
[17] P. Frutos, S. Torrado, M. E. Perez-Lorenzo, G. Frutos, A validated quantitative colorimetric assay for gentamicin, J. Pharm. Biomed. Anal., 21, 1149 (2000).
[18] T. Oja, B. Blomqvist, K. Buckingham-Meyer, D. Goeres, P. Vuorela, A. Fallarero, Revisiting an agar-based plate method: what the static biofilm method can offer for biofilm research, J. Microbiol. Methods., 107, 157 (2014).
[19] D. Jang, D. Kim, J. Moon, Influence of fluid physical properties on ink-jet printability, Langmuir, 25, 2629 (2009).

Author Biography

Mirja Palo received her MSc diploma from the University of Tartu (Estonia) in 2012. After that she started her doctoral studies in pharmaceutical sciences as a collaboration between the Åbo Akademi University (Finland) and the University of Tartu (Estonia). Her PhD research focuses on the applications of printing technologies for the personalized medicines and the solid state characterization of the printed pharmaceuticals.

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