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Rapidly dissolving bilayer microneedle arrays – A minimally invasive transdermal drug delivery system for vitamin B12

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Abstract

Vitamin B12 plays an essential role in one-carbon metabolism in the human body. A deficiency in this vitamin can lead to severe haematopoietic and neuropsychiatric disorders and is currently treated by oral or parenteral administration of exogenous vitamin. Unfortunately, the absorption of orally taken vitamin B12 is low and highly variable, while injections can cause pain and anxiety. Thus, an efficient alternative drug delivery system for overcoming these shortcomings is highly desirable. Novel polymeric microneedle (MN) arrays have the potential for minimally invasive transdermal treatment of vitamin B12 deficiency. Bilayer dissolving MN arrays (19 x 19 needles, 600 µm height) containing 135 µg vitamin B12 were cast using two different aqueous polymer blends. MN arrays showed sufficient mechanical strength for skin insertion, dissolved rapidly and delivered 72.92% of their drug load *in vitro* over 5 h. Ultimately, the potential of delivering a therapeutically relevant dose of vitamin B12 transdermally was demonstrated *in vivo* in Sprague-Dawley rats by comparison to subcutaneous injections. Maximum plasma levels of 0.37 µg/mL occurred 30 min post-MN application, highlighting the ability of fabricated MN arrays to rapidly deliver vitamin B12 transdermally.

Abbreviations: MN, microneedle; PVP, poly(vinylpyrrolidone); PBS, phosphate-buffered saline; RP-HPLC, reverse phase high performance liquid chromatography; ICH, International Conference on Harmonisation; LOQ, limit of quantification; LOD, limit of detection

Keywords

transdermal; dissolving; microneedle; vitamin B12; bilayer; poly(vinylpyrrolidone); pharmacokinetic

1. Introduction

Vitamin B12 (MW 1355.4 Da) is a water soluble vitamin also known as cobalamin, which is found in animal products such as meat and milk. Provided with a balanced diet, the recommended daily intake of 2.4 µg of this vitamin can easily be reached by healthy adults (Institute of Medicine, 1998). However, if a diet is based mainly on vegetables due to limited availability of animal products or religious or cultural beliefs, an insufficient supply of vitamin B12 is likely and will lead to a deficiency (de Benoist, 2008). Also, while a small amount of the vitamin can be absorbed passively, most of it is actively absorbed in the terminal ileum, with intrinsic factor being essential for this process.

Consequently, conditions such as ileal resection or a depletion of intrinsic factor, which is often found in elderly people, can result in malabsorption of the vitamin (Lachner *et al.*, 2012).

Vitamin B12 deficiency affects millions of people around the world and may be considered as a public health problem (Guney *et al.*, 2016). Whilst a restricted dietary intake of vitamin B12 is the most common reason for a deficiency in poor countries, with a reported prevalence of up to 80% in some populations, decreased absorption is a central cause of deficiency among the wealthier populations of the world, especially elderly people (Andrès *et al.*, 2004; Allen, 2009). Taking into consideration that the population aged over 60 years is growing faster than any other age group, an increase in the numbers of people with vitamin B12 deficiency can be expected (United Nations, 2017).

One of the active forms of vitamin B12 is methylcobalamin, which acts as a cofactor of methionine synthase and is essential for one-carbon metabolism. A deficiency can lead to haematological and neuropsychiatric symptoms, such as megaloblastic anaemia and polyneuropathy (Andrès *et al.*, 2004). It has also been associated with conditions like dementia and depression. While haematopoietic disorders can normally be reversed by supplementation of vitamin B12, neuropsychiatric symptoms are often irreversible (Andrès *et al.*, 2004). A deficiency should, therefore, be avoided or treated immediately.

Nowadays, vitamin B12 deficiency is mostly treated by subcutaneous or intramuscular injections of cyanocobalamin (Fig. 1). According to the guidelines of the *British Journal of Haematology*, patients should initially receive an intramuscular injection of 1000 µg vitamin B12 three times a week for at least two weeks until symptoms are improved (Devalia *et al.*, 2014). While a deficiency caused by dietary shortcomings can be prevented by oral substitution of vitamin B12, plasma levels in individuals with limited absorption have to be maintained by injection of 1000 µg vitamin B12 every two to three months (Devalia *et al.*, 2014). Alternatively, a high dose (1000-2000 µg) of vitamin B12 can be administered orally on a daily basis but only 1.2% of the dose is absorbed passively which can lead to treatment failure (Devalia *et al.*, 2014; Vidal-Allabal *et al.*, 2016). Excess administration of vitamin B12 is not associated with adverse effects as it is rapidly eliminated renally (Institute of Medicine, 1998). Conversely, intramuscular injections have to be administered by skilled personnel. Additionally, they do not only often cause pain and anxiety in patients, but can also lead to complications, such as haematoma, bleeding and nerve injuries if applied incorrectly (Ogston-Tuck, 2014). Considering this and the above mentioned drawbacks of orally administered vitamin B12, the development of an alternative drug delivery system is highly desirable.

Microneedle (MN) arrays are a minimally invasive and easy to handle alternative to injections (Larrañeta *et al.*, 2016). They can be easily self-administered by the patient (Ripolin *et al.*, 2017) and successful use and likely acceptability by elderly people have already been demonstrated (Quinn *et al.*, 2017). A wealth of published studies have proven that this novel drug delivery system can deliver a variety of low and high molecular weight drugs *via* the transdermal route by actively piercing through the *stratum corneum*, thus, overcoming the skin barrier (Edens *et al.*, 2015; González-Vázquez *et al.*, 2017; Hutton *et al.*, 2018; Ling & Chen, 2013; Matsuo *et al.*, 2012; McCrudden *et al.*, 2014; McCrudden *et al.*, 2015; Migalska *et al.*, 2011; Sullivan *et al.*, 2010). The MN-derived drug payload is then delivered into the viable skin layers.

MN assisted transdermal delivery of vitamin B12 could provide an excellent alternative to currently used drug delivery systems. If successful, this may lead to a considerable reduction in the numbers of people living with a vitamin B12 deficiency, as a consequence of higher acceptability of this new treatment option, when compared to current approaches. The present study explores the possibility of developing biocompatible dissolving polymeric MN arrays for transdermal delivery of a therapeutically relevant dose of vitamin B12 to potentially treat and prevent vitamin B12 deficiency. For this purpose, bilayer MN arrays with drug loaded in the MN shafts only were fabricated, characterised and tested *in vitro* and *in vivo* for delivery of vitamin B12.

2. Materials and Methods

2.1. Materials

Plasdone™ K-29/32 (poly(vinylpyrrolidone) (PVP, MW 58 kDa) and Plasdone™ K-90 D (PVP, MW 360 kDa) were donated by Ashland (Kidderminster, UK). Vitamin B12 (cyanocobalamin, purity > 98%) was purchased from Alfa Aesar (Heysham, UK). Cytamen® 1000 micrograms/mL Solution for Injection was purchased from RPH Pharmaceuticals AB (Haninge, Sweden) and Heparin Sodium (200 units in 2 mL) from Wockhardt UK Ltd (Wrexham, UK). All other chemicals used were of analytical reagent grade.

2.2. Fabrication of dissolving bilayer MN arrays

Vitamin B12 MN arrays were prepared in two steps from two different aqueous polymer blends. For MN shafts, 2.5% (w/w) vitamin B12 and 30% (w/w) PVP (MW 58 kDa) were dissolved in deionised water:ethanol (80:20, v/v). Approximately 100 mg of the blend was poured into MN moulds (19 x 19 needles). A square piece of cellulose filter paper (Whatman™, Little Chalfont, UK) was placed on top of the formulation to spread it evenly and MN moulds were wrapped in Parafilm M® (Bemis Company Inc., Soignies, Belgium), to prevent premature drying. After centrifugation at 5000 rpm for

15 min, Parafilm M®, filter paper and excess formulation were removed carefully. The first MN layer was dried at room temperature under protection from light for 4 h. For MN baseplates, drug-free aqueous hydrogels of PVP (MW 360 kDa) were prepared at various concentrations (15%, 40%, 50%, w/w). Approximately 250 mg of the hydrogel formulations were poured into MN moulds, on top of the initial dried layer and these were centrifuged at 3500 rpm for 15 min. After drying for 18 h at room temperature under protection from light, MN arrays were demoulded and sidewalls removed using a pre-heated scalpel. MN arrays were inspected visually using a Leica EZ4W stereo microscope with an integrated camera (Leica Microsystems, Milton Keynes, UK) and a Tabletop TM 3030 scanning electron microscope (Hitachi, Tokyo, Japan). They were stored in a plastic box wrapped in aluminium foil at room temperature until further use.

2.3. Determination of MN insertion and mechanical strength

A TA.XT2 Texture Analyser (Stable Micro Systems, Haslemere, UK) in compression mode was used to determine insertion characteristics into an artificial skin model and mechanical strength of bilayer MN arrays following a method developed and validated by Larrañeta *et al.* (2014). Briefly, MN arrays were attached to the cylindrical probe (cross-sectional area 1.5 cm²) of the Texture Analyser and this was moved vertically downward at a speed of 1.19 mm/s onto the artificial skin model consisting of eight Parafilm M® layers (Larrañeta *et al.*, 2014). A force of 32 N per array was applied for 30 s before MN arrays were moved upward again. Parafilm M® layers were separated and holes created in each layer were counted using the microscope. Mechanical strength of MN arrays was evaluated using a similar set up (speed 1.19 mm/s, force 32 N/array) but compressing MN arrays against a flat aluminium surface instead of a skin-simulant for 30 s. Length of individual MN tips was measured before and after compression using the stereo microscope. Percentage reduction of MN heights was calculated and reported as mean $\pm$ SD, n= 6.

2.4. Evaluation of MN in-skin dissolution kinetics

In-skin dissolution kinetics of dissolving MN arrays were evaluated using full-thickness neonatal porcine skin. This is a widely accepted skin model for human skin due to its similarities in terms of general structure, thickness, hair follicle content, pigmentation, collagen and lipid composition (Summerfield *et al.*, 2014). Skin samples were obtained from stillborn piglets. These were stored immediately after birth at -20°C and thawed for two days before being skinned. Full-thickness skin was cut into pieces and stored at -20°C until further use. Skin samples were equilibrated in phosphate-buffered saline (PBS, pH 7.4) for 30 min and shaved prior to use. Each sample was placed on tissue paper soaked in PBS to maintain skin hydration and pinned to a Styrofoam™ platform wrapped in aluminium foil. MN arrays were inserted manually into the skin using a 1 mL syringe plunger. After applying pressure for 30 s, a 5.0 g stainless-steel cylinder was placed on top of MN arrays, keeping them in place. MN arrays were removed after 30 s, 60 s and 120 s and the degree of dissolution was inspected under the stereo microscope.

2.5. Determination of vitamin B12 content in MN arrays

For evaluation of the total content of vitamin B12 in MN shafts, an accurate volume of the formulation was weighed and the density calculated dividing mass by volume. Furthermore, the volume of the conical MN shafts within one array was determined, based upon needle length and width. The total vitamin B12 content was then calculated by multiplying the relative drug content of the formulation by its density and the volume of all MN shafts within one array.

2.6. HPLC assay development and validation

For the quantification of vitamin B12 in *in vitro* and *in vivo* sample matrices, a reverse phase high performance liquid chromatography (RP-HPLC) method was developed by modifying a published assay (Vigbedor *et al.*, 2015). Analysis was performed on an Agilent 1220 Infinity LC system (Agilent

Technologies UK Ltd., Stockport, UK) with a Phenomex® Kinetex® 5 μ m C18 100 Å column (150 x 4.6 mm; Phenomenex, Cheshire, UK) and UV detection at 362 nm. The column temperature was maintained at 25°C. Separation was obtained at a flow rate of 0.6 mL/min with deionised water:methanol (72:28, v/v) as a mobile phase and a run time of 7 min per sample. The injection volume was 20 μ L for *in vitro* and 70 μ L for *in vivo* sample matrices. The RP-HPLC method was developed and validated according to the guidelines of the International Conference on Harmonisation (ICH 2005). Specificity was shown by comparing chromatograms of blank and spiked samples. Inter- and intra-day accuracy and precision were investigated and limit of quantification (LOQ) and limit of detection (LOD) were calculated based on the residual standard deviation of the regression line.

2.7. Sample preparation

In vitro samples were centrifuged at 14,800 rpm for 5 min prior to RP-HPLC analysis. To separate plasma from blood, blood samples were centrifuged at 2000 rpm for 15 min at 4°C in a refrigerated centrifuge (Sigma 2-16K, Osterode am Harz, Germany). For protein precipitation, a volume of 0.5 mL methanol was added to 100 μ L plasma and vortexed for 10 min. After centrifugation at 12,358 rpm for 10 min at 4°C, the supernatant was transferred to a glass culture tube. To achieve optimum extraction, this procedure was repeated and the second supernatant was also added to the glass culture tube. A Zymark TurboVap® LV Evaporator Workstation (McKinley Scientific, Sparta, NJ, USA) was then used for drying the extracts under streaming nitrogen over 50 min at 35°C. Samples were reconstituted in 100 μ L deionised water and centrifuged at 12,358 rpm for 15 min at 4°C prior to RP-HPLC analysis.

2.8. *In vitro* permeation study

Transdermal permeation of vitamin B12 across dermatomed neonatal porcine skin from dissolving MN arrays was investigated *in vitro* using a modified Franz-cell apparatus set up, as described

previously (McCrudden *et al.*, 2014). Skin was obtained as explained above, but using an Integra® Padgett® Electric Slimline Dermatome (Integra® LifeSciences Corporation, Plainsboro, New Jersey) for trimming the skin to a thickness of 350 µm (Garland *et al.*, 2012). The shaved skin was attached to the donor compartment of each cell with the *stratum corneum* facing upwards, using cyanoacrylate glue (Loctite®, Dublin, Ireland). Donor compartments were placed upon a sheet of dental wax covered in aluminium foil and MN arrays were inserted into the skin using a 1 mL syringe plunger applying firm pressure for 30 s. To secure them in place, a 5.0 g stainless-steel cylinder was placed on top of MN arrays. Donor compartments were attached to receiver compartments containing 12 mL PBS (pH 7.4), thermostatically maintained at $37 \pm 1^\circ\text{C}$ and stirred with a magnetic stir bar at 600 rpm. To prevent evaporation, Franz-cells were wrapped in Parafilm M®. At predetermined time points, 300 µL samples were withdrawn using 1 mL syringes attached to 8 cm needles and replaced with an equal volume of PBS. Samples were analysed using the validated HPLC method and a vitamin B12 permeation profile was constructed.

2.9. *In vivo* study

Ethical permission for all *in vivo* experiments was obtained from the Queen's University Belfast, Biological Services Unit (BSU) and all researchers carrying out the work held Personal Licences from the UK Home Office. All *in vivo* experiments were conducted according to the policy of the federation of European Laboratory Animal Science Associations and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes, with implementation of the principles of the 3Rs (replacement, reduction, and refinement).

Female Sprague-Dawley rats weighing 221.25 ± 26.94 g were used in this experiment. The rats were acclimatised to laboratory conditions for at least 7 days prior to the commencement of experiments. The animals were separated into two cohorts. The control cohort ($n = 6$) received a subcutaneous injection with a volume of 200 µL containing 200 µg vitamin B12. Blood samples were taken at

designated time points (0.5, 1, 2, 3, 24 h) *via* tail vein bleeds, subdividing the control cohort into two sampling time point groups to facilitate frequency of blood sampling.

In the second group of rats ($n = 9$), the test cohort, the animals were anaesthetised under gas anaesthesia (2-4% isoflurane in oxygen) and the hair on the backs of the animals was removed using a shaver and hair removal cream, prior to application of MN arrays. Four MN arrays (total patch size 1.44 cm^2) were applied to the back of each animal with firm finger pressure. MN arrays were secured in place for 24 h with Microfoam™ Surgical Tape (3 M, Bracknell, UK), an occlusive layer of Tegaderm™ (3 M, St Paul, Minnesota, USA) and Micropore™ Surgical Tape (3 M, Wroclaw, Poland), which was wrapped around the animals. The test cohort of rats was subdivided into three sampling time point groups to facilitate frequency of blood sampling, with blood samples taken at 0.5, 0.75, 1, 2, 3, 6, 24 and 30 h.

3. Results

3.1. Characteristics of dissolving bilayer MN arrays

Upon visual inspection using the stereo microscope, each MN array showed 361 conically shaped needles measuring heights of $600.33 \pm 2.94 \text{ } \mu\text{m}$ ($n = 6$) and widths of $300.17 \pm 2.04 \text{ } \mu\text{m}$ ($n = 6$) at the base (Fig. 2A, 1B). Scanning electron microscopy displayed clearly formed sharp needles across the whole array (Fig. 2D).

At first, MN arrays were prepared using an aqueous blend of 15% (w/w) high molecular weight PVP for casting the baseplates as this concentration had been previously used by Thakur *et al.* (2016), forming mechanically strong baseplates. However, after centrifugation, diffusion of vitamin B12 from MN shafts into the MN baseplate was observed (Fig. 2E). To prevent this, the concentration of PVP was subsequently increased to 40% (w/w) (Fig. 2F) and then to 50% (w/w) (Fig. 2G). Only MN arrays with baseplates prepared from an aqueous blend of 50% (w/w) PVP showed no signs of diffusion upon visual inspection and were, therefore, taken forward in all subsequent studies.

3.2. Determination of MN insertion and mechanical strength

To evaluate the insertion capabilities of fabricated MN arrays, they were inserted into an artificial skin model consisting of eight Parafilm M® layers (Larrañeta *et al.*, 2014), showing the ability to pierce the first three layers. The first layer was pierced by $99.8 \pm 0.3\%$ of the 361 MN shafts located on one array, the second by $95.1 \pm 1.4\%$ and the third by $20.4 \pm 8.6\%$. The percentage of holes created in each Parafilm M® layer is presented in Fig. 3. Considering an average thickness of $127 \mu\text{m}$ for each layer, MN shafts inserted down to $381 \mu\text{m}$, which equals approximately 63.5% of the total needle length of $600 \mu\text{m}$ and is similar to results obtained in previous studies (Larrañeta *et al.*, 2014). Compressing MN arrays against a flat aluminium surface resulted in a percentage reduction of MN shafts in height of $2.5 \pm 2.0\%$ ($n = 6$), indicating high mechanical strength (Fig. 2B, C).

3.3. Evaluation of MN in-skin dissolution kinetics

The rapid dissolution process of MN shafts post-insertion into full-thickness neonatal porcine skin is shown in Fig. 4. Dissolution commenced within seconds of insertion, with more than 50% of MN shafts dissolved after only 30 s. All MN shafts were completely dissolved within 120 s of application.

3.4. Determination of vitamin B12 content in MN arrays

A common practice for determining the drug content of dissolving MN arrays is to dissolve the whole array in a predefined volume of an aqueous medium and to then measure the resulting concentration using a validated analytical technique. In the present study this technique would have led to falsely high drug content due to traces of vitamin B12 in the baseplate. Therefore, an alternative method was used to give a more precise estimation. The density of the formulation used for casting the MN shafts was calculated to be approximately 1.05 mg/mm^3 . Multiplication of the density by the total volume of 5.11 mm^3 of all 361 MN shafts per array and the concentration of

vitamin B12 within the formulation resulted in a total amount of 135 µg vitamin B12 *per* 19 x 19 MN array.

3.5. HPLC assay development and validation

One HPLC calibration graph each for *in vitro* and *in vivo* sample matrices was generated. The method was specific for vitamin B12 in both matrices, as at the retention time ($R_t = 4.88$ min) of the vitamin (Fig. 5B), no peaks could be observed if samples were not spiked beforehand (Fig. 5A). Linearity was demonstrated within a range of 0.1 µg/mL to 25 µg/mL for *in vitro* samples and 0.05 µg/mL to 2.5 µg/mL for *in vivo* samples with the coefficient of determination (R^2) being 1 in both cases (Table 1). Additionally, inter-day and intra-day accuracy with mean recovery rates (RE) ranging between 98.49% and 100.74% and precision with a maximum relative standard deviation (RSD) of 4.32% were determined (Table 2).

3.6. *In vitro* permeation study

The *in vitro* delivery of vitamin B12 commenced within 2 min of MN application. After 15 min, $20.07 \pm 3.80\%$ (30 µg) and after 5 h, a total of $72.92 \pm 5.30\%$ (100 µg) of the drug load was delivered into the receiver compartment of the Franz cell (Fig. 6). These results indicate that most MN shafts successfully pierced the skin and dissolved, thus, releasing a high percentage of their drug load.

3.7. *In vivo* study

The *in vivo* pharmacokinetic plasma profiles obtained after subcutaneous injection of 200 µg vitamin B12 in the control animal cohort and after application of 4 MN arrays (1.44 cm²), containing a total of 540 µg vitamin B12, in the test cohort are presented in Fig. 7A & 7B, respectively. These profiles were used for calculating the drug pharmacokinetic parameters reported in Table 3. In both cases, the time taken to achieve peak plasma concentrations was 30 min (T_{max}). The maximum plasma concentration (C_{max}) of vitamin B12 post-subcutaneous administration was 1.30 ± 0.25 µg/mL and,

therefore, approximately 3.5 times higher than the maximum concentration of $0.37 \pm 0.04 \mu\text{g/mL}$ reached post-transdermal application *via* MN arrays. The mean plasma concentration dropped rapidly within 2 h for both dosage forms. While no drug was detectable after 24 h in those animals receiving the subcutaneous injection, the vitamin could still be detected in individual animals treated with MN arrays even after 30 h.

The area under the curve ($AUC_{0-30\text{ h}}$) post-subcutaneous injection was $3.01 \mu\text{g/mL}\cdot\text{h}$, whereas it was $0.81 \mu\text{g/mL}\cdot\text{h}$ for the transdermal delivery route. Considering the dose difference between the two application forms, the relative bioavailability (F_{rel}) was calculated for better comparison using the following equation:

$$F_{\text{rel}} = \frac{AUC_{0-30\text{ h}}(MN) \cdot \text{Dose}(s.c.)}{AUC_{0-30\text{ h}}(s.c.) \cdot \text{Dose}(MN)}$$

The relative bioavailability was found to be 0.1, suggesting that the amount of vitamin B12 delivered after application of 4 MN arrays was approximately $54 \mu\text{g}$.

4. Discussion

Transdermal drug delivery systems can improve patient compliance considerably as they have several advantages in comparison to other commonly used application forms (Kaestli *et al.*, 2008). However, to date, only a small number of therapeutic agents are available for transdermal delivery (Szunerits & Boukherroub, 2018). This is mainly due to the nature of skin. Its outer layer, the *stratum corneum*, forms a fierce barrier against external influences and only a very limited number of molecules with specific physicochemical properties (lipophilic, molecular weight < 500 Da) are able to pass across it. Therefore, most topically applied drugs cannot permeate through the skin to reach the underlying tissues and systemic blood circulation (Williams, 2003).

Polymeric MN arrays are a novel technology with the potential to revolutionise the prospects of transdermal drug delivery (Tekko *et al.*, 2017). They are composed of arrays of a certain number of

micron-sized polymeric needles. The holes created by these arrays post-application to the skin enable therapeutic agents incorporated within the arrays to bypass the *stratum corneum* and reach the dermal microcirculation. Several studies have shown that MN arrays have the capability to increase the range of drugs that can be delivered transdermally without being affected by their physicochemical properties (González-Vázquez *et al.*, 2017; Hutton *et al.*, 2018; Migalska *et al.*, 2011; McCrudden *et al.*, 2014, 2015). Due to the short length of individual MNs, dermal nerves are not stimulated during insertion. Consequently, MNs are considered to be pain-free, minimally invasive devices. They combine the advantages of both traditional transdermal drug-delivery systems, in terms of patient compliance and the delivery capabilities of conventional hypodermic needles (Tekko *et al.*, 2017).

It has been shown that Vitamin B12 is barely able to bypass the *stratum corneum* without further enhancement techniques, owing to its size (MW 1355.4 Da) (Yang *et al.*, 2011). Therefore, the aim of this study was to develop bilayer rapidly dissolving MN arrays with sufficient mechanical strength for successful skin insertion and the ability to deliver a therapeutically relevant dose of vitamin B12 transdermally.

Due to its biocompatibility, non-toxicity and high water-solubility, PVP (MW 58 kDa) was chosen to fabricate the shafts of the dissolving MN arrays. This polymer is generally regarded as safe by the US Food and Drug Administration (FDA) and is widely used in injections and as a blood volume expander (Ravin *et al.*, 1952). A further advantage is that low molecular weight PVP can be cleared by the kidneys and, therefore, does not accumulate in the body even after several applications (Ravin *et al.*, 1952). To obtain MN baseplates with high mechanical strength, a higher molecular weight PVP (MW 360 kDa) was chosen. A concentration of 15 % (w/w) PVP was previously shown to form strong baseplates (Thakur *et al.*, 2016). However, upon preparation of bilayer MN arrays using this concentration of the selected polymer, considerable amounts of vitamin B12 migrated from MN shafts into baseplates. As one of the aims of this study was to concentrate vitamin B12 into the MN

shafts only, the amount of PVP used for baseplate fabrication was subsequently increased to 50%. Consequently, diffusion of drug from MN shafts into the baseplate was prevented and two clearly separated layers, namely MN shafts and baseplate were formed.

The formulation of bilayer MN arrays has many advantages. Different characteristics for MN shafts and baseplates, in terms of mechanical strength and dissolution kinetics can be achieved. Furthermore, the location of the therapeutic agent into the shafts only, leads to a reduction in drug waste. This is especially beneficial in scale-up processes and for highly expensive agents. For the preparation of bilayer MN arrays, different techniques have been performed previously (Fukushima *et al.*, 2011; Ling *et al.*, 2013; Zaric *et al.*, 2013; Wang *et al.*, 2015; McCaffrey *et al.*, 2016; González-Vázquez *et al.*, 2017; Vora *et al.*, 2017). While González-Vázquez *et al.* (2017) placed a pre-formed baseplate onto cast MN shafts, Vora *et al.* (2017) prepared a suspension containing nanoparticles and then concentrated them into MN shafts by high speed centrifugation. As these methods were not applicable in the present study, a new technique was developed for forming two different layers. The fabricated bilayer MN arrays were characterised *in vitro* in terms of their mechanical strength, insertion into skin, dissolution kinetics in skin, and their capability to deliver their drug load in *in vitro* and *in vivo* settings. Although, compressing MN arrays against an aluminium block using a texture analyser is not similar to MN insertion into skin, this test is a widely accepted method for evaluating their mechanical strength (McCrudden *et al.*, 2014; Thakur *et al.*, 2016; Ripolin *et al.*, 2017). In the present study, a constant force of 32 N per array was applied for 30 s as this equates to the force used by humans for successful insertion of MN arrays (Larrañeta *et al.*, 2014). The calculated reduction of MN shaft heights was minimal ($2.5 \pm 2\%$, $n=6$). Previous studies showed that MN arrays which encountered up to 20% reduction in height were still successfully inserted into skin in *in vitro* and *in vivo* settings (McCrudden *et al.*, 2014; Thakur *et al.*, 2016; Ripolin *et al.*, 2017). Therefore, the obtained test results indicate that the fabricated MN arrays have sufficient mechanical strength for insertion into human skin. Additionally, they inserted excellently into an artificial skin simulant

consisting of Parafilm M® (Larrañeta *et al.* 2014). Application of MN arrays to neonatal porcine skin, a widely accepted model for human skin, demonstrated the desired rapid dissolution of the needle shafts *in vitro*. More than 50% of MN shafts dissolved within 30 s of application, with all shafts dissolved after only 2 min, thus reducing application time to a minimum. These results agree with data previously obtained by Thakur *et al.* (2016) who reported a dissolution time of 3 min in ocular tissues for the same polymer. Before carrying the developed bilayer MN arrays forward to an *in vivo* study, the ability to deliver vitamin B12 through neonatal porcine skin was demonstrated *in vitro* using Franz-cells. More than 70% of the incorporated drug load was released within 5 h.

The *in vivo* study clearly demonstrated that the fabricated bilayer MN arrays can deliver vitamin B12 transdermally in a minimally invasive way. Upon removal of the MN array patches, MN shafts were completely dissolved and no erythema was observed at the application site. Any remaining baseplates could be easily wiped off the skin with a wet cloth. Maximum blood levels were reached within 30 min post-application of the patches to the back of the animals. It should be noted, that the AUC_{0-30 h} after subcutaneous injection was 3.7 times higher compared to MN application, even though the applied patches contained 2.7 times more drug. However, this can be explained by the fact that only the inserted part of MN shafts (63.5% in *in vitro* settings) would be expected to release their drug load. Considering that vitamin B12 is commonly applied intramuscularly or subcutaneously, the AUC post-subcutaneous injection can be assumed to present the maximal delivery possible. Under this assumption, the relative bioavailability of vitamin B12 from MN patches was calculated to be 0.1 compared to subcutaneous delivery. The amount of vitamin delivered transdermally with a patch size of 1.44 cm² was therefore approximately 54 µg.

This study represents a proof of concept for the feasibility of using rapidly dissolving MN arrays for transdermal delivery of vitamin B12. Predicting pharmacokinetics in humans on the basis of animal studies requires further investigation, especially as pharmacokinetic results for elderly people presented by Tillemans *et al.* (2014) demonstrated considerable differences compared to results

obtained in the present study. Nevertheless, the patch size necessary to deliver therapeutically relevant amounts of vitamin B12 can be cautiously extrapolated from the data presented here. Considering that a patch sized 1.44 cm² delivered approximately 54 µg, a patch size of approximately 27 cm² might be sufficient to deliver the commonly applied dose of 1000 µg. This area could be reduced by increasing the drug load within the MN arrays. As vitamin B12 is only sparingly water soluble, formulating a nanosuspension prior to casting MN arrays is also a possible approach (Vora *et al.*, 2018; McCrudden *et al.*, 2018). Although there are considerable inherent differences between MN patches and currently marketed transdermal patches, relatively big patches are generally accepted by patients. Even without further reduction in the MN patch size determined here, the calculated area of 27 cm² is well within the range of available transdermal patches, with Nicotinell® (nicotine) patches measuring 30 cm² (Novartis, 2018) and Duragesic® CII (fentanyl) patches measuring 32 and 42 cm² (Janssen, 2018). MN patches sized up to 25 cm² have already been prepared previously (Donnelly *et al.*, 2012) and the successful application of large patches by human volunteers has been shown (Ripolin *et al.*, 2017). Therefore, even though further investigations are required, the results obtained in this study show the potential of delivering clinically relevant doses of vitamin B12 using dissolving polymeric MN arrays.

5. Conclusion

This study reports the successful incorporation of vitamin B12 into bilayer dissolving polymeric MN arrays. Moreover, it demonstrates that the transdermal delivery of a therapeutically relevant dose of vitamin B12 *in vivo* within a short period of time is feasible by using these novel devices. This highlights the promising impact this minimally invasive technology could have on the future treatment of vitamin B12 deficiency caused by malabsorption. Further studies are required however before MN arrays can be successfully taken forward for the potential treatment of vitamin B12 deficiency. Specifically, the design, formulation and fabrication of larger MN patches; up-scaling the manufacture of MN arrays in an economically viable way and the introduction of standardised

mechanical and characterisation tests for MN arrays are all necessitated before this drug delivery platform can be used to effectively treat those people affected by vitamin B12 deficiency.

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Figure 1. Chemical structure of cyanocobalamin.

Figure 2. (A) Light microscope image (10x) of a dissolving bilayer MN array with vitamin B12 incorporated only into the needle shafts. The array was cast using a mould with 361 conical shaped needles (19 x 19), measuring 600 μm in height and 300 μm at base. (B) Light microscope image (35x) showing MN shaft height measurements of a MN array before compression. (C) Light microscope image (35x) showing MN shaft height measurements after compression (32 N/array, 30 s). (D) Scanning electron microscope image (150x) displaying surface characteristics of MN shafts. (E-G) Light microscope images of bilayer MN arrays with three different baseplates cast out of hydrogels made from aqueous blends containing 15% (w/w) (E), 40% (w/w) (F) and 50% (w/w) PVP (MW 360 kDa) (G), respectively.

Figure 3. Percentage holes created in each Paraform M® layer (means $\pm$ SD, $n = 3$) by insertion of MN arrays with 361 needles of 600 μm heights into the Paraform M® skin simulant.

Figure 4. Light microscope images of MN arrays following insertion into neonatal porcine skin and removal after predetermined time periods of 30 s, 60 s and 120 s.

Figure 5. HPLC chromatograms after injecting (A) blank rat plasma and (B) a sample prepared from rat plasma spiked with 2.5 µg/mL vitamin B12 ($R_t = 4.88$ min, highlighted in red box).

Figure 6. *In vitro* permeation profile of vitamin B12 through dermatomed neonatal porcine skin (means $\pm$ SD, $n = 3$).

Figure 7. (A) *In vivo* plasma profile of vitamin B12 after subcutaneous injection of 200 µL aqueous vitamin B12 solution (200 µg) (means $\pm$ SD, $n = 3$ at 0.5, 1, 2, 3 h and $n = 6$ at 24 h). (B) *In vivo* plasma profile of vitamin B12 after application of 4 MN arrays containing a total of 540 µg vitamin B12 (means $\pm$ SD, $n = 2$ at 3 h, $n = 3$ at 0.5, 0.75, 1, 2, 6 h and $n = 6$ at 24, 30 h).

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Table 1. Summary of the calibration parameters for HPLC analysis of vitamin B12 in *in vitro* and *in vivo* sample matrices. Presented are slopes, y-intercepts, coefficients of determination (R^2), ranges, limits of quantification (LOQ) and limits of detection (LOD).

	Slope	y-Intercept	R^2	Range [$\mu\text{g/mL}$]	LOQ [$\mu\text{g/mL}$]	LOD [$\mu\text{g/mL}$]
<i>In vitro</i>	33.315	-0.5109	1	0.1 - 25	0.049	0.016
<i>In vivo</i>	101.07	-0.4411	1	0.05 - 2.5	0.038	0.012

Table 2. Summary of the inter-day and intra-day accuracy and precision of HPLC analysis of vitamin B12 in *in vitro* and *in vivo* sample matrices as represented by theoretical concentrations (C_T) covering the range of the methods, calculated concentrations (C_C , means $\pm$ SD, $n = 3$), percentage recoveries (RE) and relative standard deviations (RSD).

		Inter-day			Intra-day		
	C_T [$\mu\text{g/mL}$]	C_C [$\mu\text{g/mL}$]	RE [%]	RSD [%]	C_C [$\mu\text{g/mL}$]	RE [%]	RSD [%]
<i>In vitro</i>	1	1.01 $\pm$ 0.03	100.59	3.32	0.98 $\pm$ 0.04	98.49	4.32
	10	10.00 $\pm$ 0.05	100.04	0.45	10.00 $\pm$ 0.01	99.96	0.10
	20	19.90 $\pm$ 0.36	99.50	1.81	20.15 $\pm$ 0.03	100.74	0.14
<i>In vivo</i>	1	0.99 $\pm$ 0.03	98.49	3.05	1.00 $\pm$ 0.01	99.61	1.05
	2	1.99 $\pm$ 0.04	100.45	1.95	2.01 $\pm$ 0.00	100.35	0.13
	2.5	2.50 $\pm$ 0.01	99.47	0.52	2.50 $\pm$ 0.02	100.00	0.66

Table 3. Pharmacokinetic parameters for vitamin B12 obtained in rats after subcutaneous (s.c.) injection or application of 4 MN arrays. Parameters displayed are area under the curve ($AUC_{0-30 \text{ h}}$), peak plasma concentration (C_{max} , means $\pm$ SD, $n = 3$) and time to peak plasma concentration (T_{max}).

	s.c.	MN
$AUC_{0-30 \text{ h}}$ [$\mu\text{g/mL}\cdot\text{h}$]	3.01	0.81
C_{max} [$\mu\text{g/mL}$]	1.30 $\pm$ 0.25	0.37 $\pm$ 0.04
T_{max} [min]	30	30

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: