Nanoscale

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Cite this: *Nanoscale*, 2018, **10**, 9773

Ultra-low voltage triggered release of an anti-cancer drug from polypyrrole nanoparticles†

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Received 11th February 2018,
Accepted 6th May 2018

DOI: 10.1039/c8nr01259h

rsc.li/nanoscale

We have synthesized polypyrrole nanoparticles using three different oxidizing agents (hydrogen peroxide, chloroauric acid and ferric chloride) and shown that films assembled from these nanoparticles have significantly different drug release profiles. When ferric chloride is used as the oxidizing agent, it is possible to release drugs at voltages as low as −0.05 V, almost an order of magnitude lower than typically used voltages. These ultra-low voltage responsive nanoparticles widen the window of operation of conducting polymers and enable delivery of redox active drugs. As an example, we have shown pulsed release of the chemotherapeutic methotrexate at voltages as low as −0.075 V, demonstrating the potential application of these nanoparticles in cancer treatment. We have also verified the anti-tumor efficacy of the released drug using PC12 cell cultures.

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Introduction

Electroresponsive polymers are attractive materials for designing spatiotemporally controlled drug delivery systems.[1,2] These materials can release drugs on demand in response to an externally applied electric stimulus and can be used to build programmable drug delivery devices.[3] The quantity of drug discharged can be precisely controlled by fine tuning the electrical parameters. This level of control is important for delivery of drugs which have a narrow therapeutic window to prevent toxicity. Moreover, localized delivery can diminish systemic drug distribution, thereby reducing overall drug dosage, cost, and side effects.

Over the past few decades, there has been significant interest in the development of novel electroresponsive materials for biomedical applications. Electroactive hydrogels[1] and conducting polymers[2] have been shown to release various drugs under appropriate electrical stimulation. Although hydrogels provide better biocompatibility and biodegradability compared to conducting polymers, they often require large currents (0.5–5 mA) or voltages (2–25 V) for their operation.[1] In contrast, conducting polymers require much lower voltages (0.5–3 V) for drug release.[2]

The most widely studied conducting polymer is polypyrrole.[2] Drug release is evoked by partial oxidation or reduction of the polymer backbone. Prior research has shown that drugs with significantly different properties such as dexamethasone,[4] doxorubicin,[5] risperidone,[6] neurotrophin-3[7] and insulin[8] can be released from electrochemically grown thin films of the polymer. In recent years, the focus has shifted to developing nanostructured films with enhanced drug loading. Nanoporous films prepared using colloidal templates[9–11] as well as nanowire-based films prepared using anodic aluminum oxide templates[5,12] have shown ~10 fold increase in drug loading capacity as well as drug release per stimulus compared to conventional flat films.

In our group, we have developed nanoparticles of polypyrrole for electroresponsive drug delivery.[13,14] Compared to films, nanoparticles allow easier scalability, greater processability and improved drug loading. In our previous work, we have shown that in certain cases, it is possible to attain drug loading as high as 51 wt%.[15] However, for small molecule drugs, which are encapsulated within the nanoparticles, only 10% of the incorporated drug could be released.[14] Moreover, the reduction process for nanoparticles in solution is limited by the diffusion of the nanoparticles from the bulk to the surface of the electrode.[16] Another challenge associated with conducting polymers in general is incompatibility with electroactive drugs; these drugs may be degraded on application of voltages necessary for drug release.[2,17]

To harness the advantages of both films and nanoparticles, in this work, we have drop-casted polypyrrole nanoparticles onto a carbon working electrode to fabricate a nanoparticle-assembled film (NAF). In prior studies, we had synthesized polypyrrole nanoparticles (PPy NPs) in a one-step microemulsion technique using hydrogen peroxide as the oxidizing

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† Electronic supplementary information (ESI) available: DLS and SEM images of FePPy and AuPPy NPs, CV data, and SEM images of NAFs. See DOI: 10.1039/c8nr01259h

‡ Equal contribution.

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agent.[14,15] It is well-known that the size, shape and electrochemical properties of polypyrrole are dependent on the oxidant used.[18,19] Therefore, here, we have performed a comparative study by preparing polypyrrole nanoparticles with three different oxidizing agents, namely, hydrogen peroxide (H_2O_2), chloroauric acid ($HAuCl_4$) and ferric chloride ($FeCl_3$) to investigate their effect on drug release. Our aim was to improve drug release per stimulus and thereby, reduce the voltage required to trigger drug release. We initially used fluorescein sodium salt (FL) as a model compound for ease of visualization and quantification. Our results show that the morphology as well as the drug release properties of the NAFs are dependent on the oxidant used in the nanoparticle synthesis.

After determination of the optimal conditions for drug release from these NAFs, we have tested the release of methotrexate (MTX) as a model chemotherapeutic agent. Methotrexate is considered to be an essential medicine by the World Health Organization[20] and is used in the treatment of several different types of cancer (breast, skin, lung, *etc.*) as well as in the treatment of some autoimmune diseases (psoriasis, rheumatoid arthritis, *etc.*).[21–23] Electroresponsive delivery would be particularly beneficial for anti-cancer treatment as it can (i) provide localized delivery to the tumor site, reducing off-target side effects, (ii) increase patient adherence to medication through long-term programmed dosage, and (iii) enable adjustable dosing to maintain the drug concentration within the therapeutic window. Our results show that more than 70% of the incorporated methotrexate can be released in a linearly pulsed manner and that voltages as low as −0.075 V *vs.* Ag/AgCl can evoke drug release. We have confirmed the anti-tumor efficacy of the released methotrexate in PC12 cell lines.

Materials

Pyrrole (reagent grade, 98%), sodium dodecyl sulfate (ReagentPlus®, ≥98.5%), 35 wt% hydrogen peroxide H_2O_2, piroxicam (>98%), fluorescein sodium salt, chitosan, methotrexate, iron(III) chloride (reagent grade), MTT Cell Growth Assay Kit and gold(III) chloride trihydrate were purchased from Sigma Aldrich. Screen printed electrodes were purchased from Metrohm and Pine Instruments. EMD Millipore Amicon™ Ultra-0.5 Centrifugal Filter Units (100 kDa MWCO) were purchased from Fisher Scientific. Cell culture medium and reagents including Dulbecco's Modified Eagle Medium (DMEM), heat-inactivated fetal bovine serum, and horse serum were purchased from Life Technology (Rockville, MD).

Methods

FL release from PPy NPs coated with chitosan (drug loaded outside the nanoparticles)

All reactions and measurements were carried out in triplicate and at room temperature, unless mentioned otherwise. To a stirring solution of 500 μL of 0.1 M SDS in 40 mM HCl, 2.5 μL of pyrrole were added. 40 μL of 625 mg mL^{-1} $FeCl_3$ or 30 μL of 625 mg mL^{-1} $HAuCl_4$ + 10 μL water were added to initiate the oxidation. In case of H_2O_2, 10 μL pyrrole and 20 μL H_2O_2 were used. The reactions were stirred for 24 h.

On a Pine screen printed electrode (SPE) with a carbon working electrode (4 × 5 mm^2), carbon counter electrode and Ag/AgCl reference electrode, a mixture containing 5 μL of PPy NPs prepared with $FeCl_3$ or $HAuCl_4$ containing 3.75 μg FL were drop-casted. In the case of H_2O_2, 1.25 μL PPy NPs and 2.5 μL 1 M NaCl were used. 5 μL of 0.05 wt% chitosan in 0.1 M HCl were drop-casted over the PPy NPs. The electrodes were then soaked in 10 mL water for 24 h.

100 μL of the water solution were placed in a well of a 96-well plate containing 100 μL 0.1 N NaOH. The absorbance of the resultant solution between 400–700 nm was read using a TECAN infinite M1000 plate reader. From the absorbance maxima, the amount of FL leaked was calculated using a calibration curve. From this value and the initial amount of fluorescein added, the amount of FL incorporated into the film was calculated.

400 μL 0.9 wt% NaCl were applied to the SPE, covering all electrodes, and 5 stimuli of −0.25 V *vs.* Ag/AgCl for 100 s were applied. An aliquot of 100 μL of the solution was retrieved from each electrode. Thereafter, −1 V was applied for 20 s, and another 100 μL of solution was retrieved. Control experiments were performed without applying voltage. 0.1 N NaOH was added to the retrieved solutions in a 1 : 1 ratio. The absorbances of the resultant solutions were read and the amount of FL released was calculated using a calibration curve.

Pulsed release of FL from PPy NPs (drug loaded inside the nanoparticles)

Nanoparticles were synthesized as described above except 6.25 μL 60 mg mL^{-1} FL were added to the reaction mixture before the addition of $FeCl_3$ or $HAuCl_4$.

On a Metrohm SPE with a carbon working electrode (3 mm diameter), carbon counter electrode and Ag/AgCl reference electrode, a 3 μL aliquot of the PPy NPs prepared above were drop-casted. The SPEs were soaked in 2 mL water for 24 h. The amount of FL leaked in the water was measured spectrophotometrically; the drug loading was calculated as mentioned above.

The SPE was immersed in a custom-designed low volume cell containing 400 μL 0.9 wt% NaCl. 5 stimuli of −0.5 V *vs.* Ag/AgCl for 20 s were applied every 3 min. An aliquot of 200 μL of the saline solution were retrieved, mixed with 200 μL 0.1 N NaOH and filtered at 10 000 rpm in an Eppendorf Centrifuge 5415 C for 2 min using a 100 kDa centrifugal filter. 200 μL of the filtrate was used to measure absorbance and quantify the amount of FL released. The cell was replenished with 200 μL of fresh saline solution in between successive stimuli. Control experiments were performed without applying voltage. The purpose of the centrifugal filter was to remove any ferric hydroxide precipitate formed from the reaction between NaOH and left-over $FeCl_3$.

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Low voltage FL release (drug loaded inside the nanoparticles)

5 μL of FL loaded PPy NPs prepared using $FeCl_3$ or $HAuCl_4$ were dropcasted on a Pine SPE with a carbon working electrode (4 × 5 mm^2). The electrodes were soaked in 10 mL water for 24 h. The amount of FL leaked was monitored spectrophotometrically and the amount of FL incorporated was calculated. Thereafter, 400 μL of 0.9 wt% NaCl solution was applied to the SPE. Two sets of experiments were done using two stimulation voltages: (i) 3 stimuli of −0.25 V of different duration (100 s, 100 s, and 300 s) and (ii) 250 stimulations of −0.05 V for 10 s each were applied. Control experiments were performed in which the electrodes were immersed in the saline solution, but no voltage was applied. At the end of the stimulations, the amount FL released was measured spectrophotometrically using 100 μL of the saline solution, added to 100 μL of 0.1 N NaOH.

Pulsed MTX release from PPy NPs

To a stirring solution of 500 μL of 0.1 M SDS in 40 mM HCl, 1 mg MTX and 2.5 μL of pyrrole were added. 40 μL of 625 mg mL^{-1} $FeCl_3$ were added to initiate oxidation. The reaction was stirred for 24 h.

On a Metrohm SPE with a carbon working electrode (3 mm diameter), carbon counter electrode and Ag/AgCl reference electrode, 3 μL of the PPy NPs prepared above were dropcasted. The SPEs were soaked in 2 mL distilled water for 24 h. The amount of MTX leaked in the water was measured spectrophotometrically using 150 μL of the sample added to 150 μL 0.1 N NaOH. The drug loading was calculated using a calibration curve.

The SPE was immersed in a custom-designed low volume cell containing 300 μL 0.1× PBS (1× PBS diluted with 0.9 wt% NaCl). Electric stimulations were applied 3.5 minutes apart. Each stimulation consisted of 5 pulses of −0.5 V *vs.* Ag/AgCl for 20 s. After 2 minutes from the start of each stimulation, 150 μL of the electrolyte were sampled and mixed with 150 μL 0.1 N NaOH for spectrophotometric quantification of drug release. The cell was replenished with 150 μL of fresh electrolyte solution in between successive stimuli. Control experiments were performed without application of voltage.

Low voltage MTX release

The Metrohm SPE as prepared above was immersed in a custom-designed low volume cell containing 300 μL 0.1× PBS (1× PBS diluted with 0.9 wt% NaCl). 125 stimulations of −0.075 V *vs.* Ag/AgCl for 10 s were applied. 150 μL of the electrolyte were sampled and mixed with 150 μL 0.1 N NaOH for spectrophotometric quantification of drug release. Control experiments were performed without application of voltage.

Measurement of size of PPy NPs

The size of PPy NPs were measured using dynamic light scattering (DLS, Zetasizer Nano ZS90 instrument), scanning electron microscopy (SEM, Zeiss Sigma FESEM) and transmission electron microscopy (TEM, FEI Tecnai G2 F20 X-TWIN).

MTX bioactivity test with PC12 cell cultures

On a Metrohm SPE with a carbon working electrode (3 mm diameter), carbon counter electrode and Ag/AgCl reference electrode, 3 μL of the PPy NPs prepared above were dropcasted. The SPEs were soaked in 2 mL distilled water for 24 h. The amount of MTX leaked in the water was measured spectrophotometrically using 150 μL of the sample added to 150 μL 0.1 N NaOH. The drug loading was calculated using a calibration curve.

The SPE was immersed in a custom-designed low volume cell containing 150 μL 0.1× PBS (1× PBS diluted with 0.9 wt% NaCl). 3 stimulations were applied 3.5 minutes apart. Each stimulation consisted of 5 pulses of −0.5 V *vs.* Ag/AgCl for 20 s. 120 μL of the electrolyte was retrieved and the amount of drug released was quantified. Three control experiments were performed: (1) without application of voltage, (2) with voltage on bare PPy NPs, and (3) without voltage on bare PPy NPs.

PC12 cells were purchased from American Type Culture Collection (Manassas, VA). The cells were cultured in DMEM supplemented with 5% horse serum and 5% fetal bovine serum in T25 cell-culture flasks. After the cells reached confluence, 2 mL accutase was added to the cells to retrieve the cells. Wells of a 96-well plate were seeded with 5 μL of the cell-suspension. 5 μL of the electrolyte in contact with PPy NPs and 70 μL DMEM were added. After 68 h, an MTT assay was performed to determine cell viability.

Graphs and figures

The data points plotted on all the graphs are the averages of $n = 3$ replicate measurements, unless mentioned otherwise. Error bars correspond to one standard deviation.

Results and discussion

Synthesis of FL-loaded polypyrrole nanoparticles

Polypyrrole nanoparticles were synthesized in a one-step microemulsion technique. To a micellar solution of sodium dodecyl sulfate, pyrrole was added, followed by addition of the oxidizing agents. Three different oxidizing agents were used, namely, H_2O_2, $HAuCl_4$, and $FeCl_3$, and the corresponding PPy NPs are designated as HPPy, AuPPy and FePPy. The size of the nanoparticles was measured by DLS, TEM, and SEM. DLS sizes were ~20 nm, 70 nm, and 120 nm for HPPy, AuPPy, and FePPy, respectively. From Fig. 1a, it can be seen that the DLS size of HPPy is in agreement with the size measured from TEM as well as the size reported previously.[15] While individual particles could not be identified using TEM for AuPPy and FePPy, the DLS data confirm the presence of nanoparticles (Fig. S1c and d†). Moreover, it was possible to identify the nanoparticles using SEM (Fig. S1a and b†). The size of the nanoparticles measured by SEM was ~55 nm for FePPy and ~13 nm for AuPPy. The discrepancy between the DLS and SEM sizes can be attributed to the tendency of the nanoparticles to aggregate.[13]

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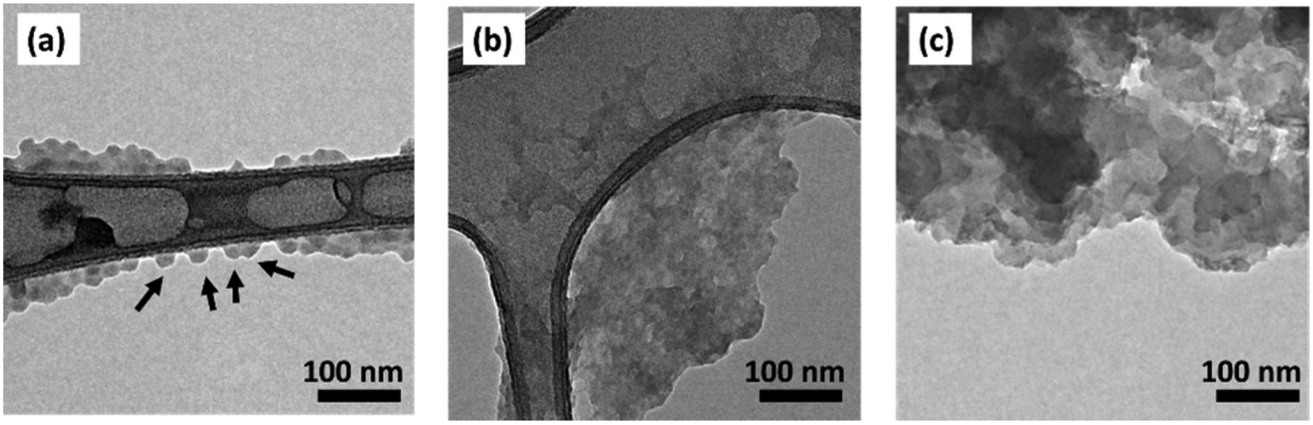

Fig. 1 TEM images of (a) HPPy, (b) AuPPy, and (c) FePPy. Arrows indicate individual particles observed in (a).

Comparison of drug release from PPy NAFs prepared with different oxidizing agents

To compare the effect of the oxidizing agent used in the synthesis of PPy NPs on the release of molecules from NAFs, the PPy NPs formed were mixed with FL and dropcasted onto a carbon working electrode of a screen-printed electrode (SPE) to form a NAF. The thickness of the NAFs varied between 10–20 μm, as observed by SEM (Fig. S2†). HPPy NPs with FL (FL-HPPy) are very well dispersible in water, and therefore, to keep them attached to the electrode, a thin layer of chitosan was dropcasted over the nanoparticles. For consistency, chitosan layers were also dropcasted over FePPy and AuPPy with FL (designated as FL-FePPy and FL-AuPPy, respectively).

Using an isotonic solution of 0.9 wt% NaCl as electrolyte, drug release experiments were carried out. Typically, voltages used for evoking drug release from polypyrrole range between 0.5–3 V.[2] We observed drugs can be released from NAFs using substantially lower voltages. It should be noted that negatively charged molecules (*e.g.*, FL or MTX) can be released by partial reduction of the polymer backbone induced by negative voltages or currents.[14,15] Initially, we stimulated the NAFs with 5 pulses of −0.25 V for 100 s, and then with 1 pulse of −1 V for 20 s. The absolute amount of FL released from FL-HPPy is negligible at −0.25 V for 100 s (Fig. 2a). In contrast, a significant amount of FL can be released from FL-FePPy. However, the different amounts of FL incorporated into the different films (Fig. 2b) should also be taken into account, and therefore, the percentage of incorporated FL released (Fig. 2c) should also be compared. Again, we observed that while less than 10% of the incorporated FL could be released from HPPy under the given stimulation conditions, ~40% could be released from the FL-FePPy NAFs. These results suggest that the oxidizing agent used in PPy NP synthesis plays an important role in drug release.

Pulsed and ultra-low voltage triggered drug release

An important aspect of an electrically controlled drug delivery system is the ability to release drugs in a pulsed manner with repeated stimuli. We further investigated the effect of oxidizing agents on drug release, focusing on FL-AuPPy and FL-FePPy. We could not release more than 10% FL from NAFs composed of FL-HPPy under any condition tested; therefore, we did not pursue further studies with those.

The amount of drug incorporated and released depends on whether the drug is adsorbed on the surface of the nanoparticles or encapsulated inside. For the subsequent tests, FL was added *in situ* during the polymerization of pyrrole. No chitosan layer was used.

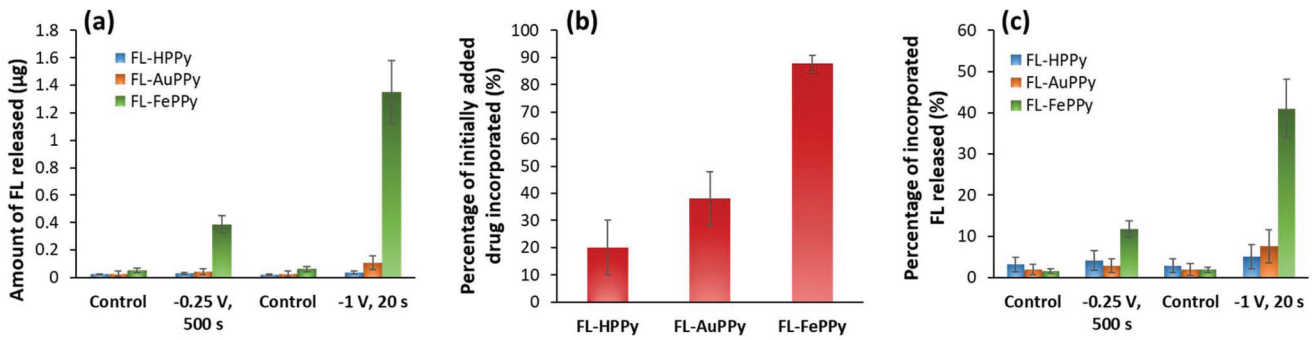

Fig. 2 Comparison of FL release from PPy NPs prepared using different oxidizing agents.

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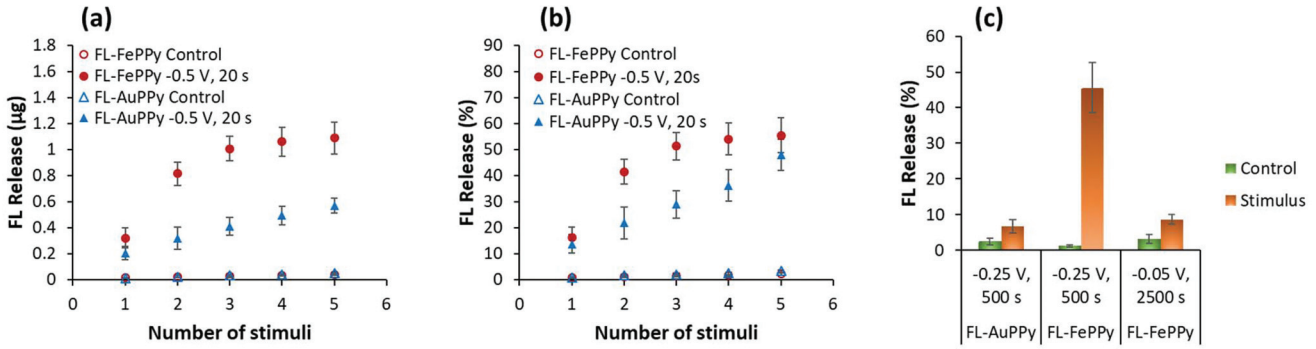

Fig. 3 (a) and (b) Comparison of FL release from FL-AuPPy and FL-FePPy; (c) low-voltage triggered FL release.

When subjected to −0.5 V for 20 s, pulsed release of FL was possible from these NAFs (Fig. 3a and b). As the amount of drug released per stimulus is greater for FL-FePPy than for FL-AuPPy, we used the former to investigate the possibility of releasing drugs using lower voltages. Long stimulation times were used for the lower voltages so that drugs could be released in amounts sufficient for quantification. We found that using both FL-AuPPy and FL-FePPy, FL can be released at −0.25 V *vs.* Ag/AgCl. However, ∼45% of the incorporated FL was released from FL-FePPy within 500 s of stimulation in comparison to ∼7% released from FL-AuPPy. These results encouraged us to study the possibility of releasing drugs at even lower voltages from FL-FePPy. We observed statistically significant FL release even at −0.05 V *vs.* Ag/AgCl from FL-FePPy, almost an order of magnitude lower than typically used voltages. We do not fully understand this behavior. No significant differences between cyclic voltammograms for FePPy and AuPPy were observed at a scan rate of 0.1 V s^{-1} between −0.6 V to +0.6 V (Fig. S3†). One potential explanation could be the difference in film morphology. It can be seen

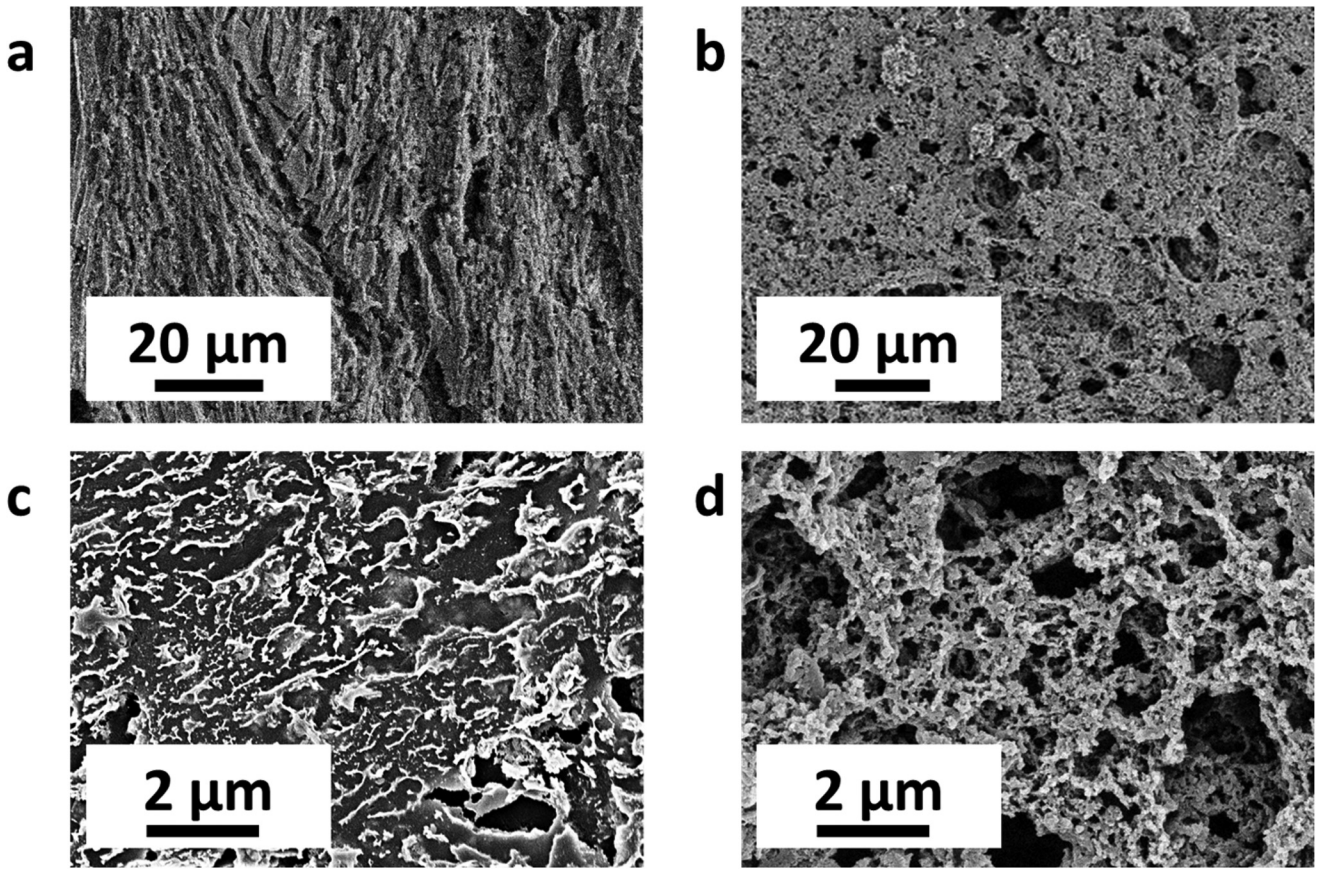

Fig. 4 SEM images of NAF formed from (a) FL-AuPPy and (b) FL-FePPy.

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from the SEM images in Fig. 4 that FL-FePPy NPs, which are larger in size, form a more porous NAF compared to the smaller FL-AuPPy NPs, which form a more compact film. Larger pore sizes in FL-FePPy NAFs perhaps facilitate the escape of FL at a faster timescale from the FePPy film compared to the AuPPy film. Due to the small dimensions of these films, however, nitrogen adsorption studies could not be performed for detailed analysis of porosity. Regardless, these data show that FePPy NAFs could be promising for developing power-efficient, ultra-low voltages responsive drug delivery devices. One of the main limitations of electroactive drug delivery systems is incompatibility with redox-sensitive drugs. The capability of releasing drugs at lower voltages expands the types of drugs that can be delivered using electrically controlled systems. Additionally, the lowest voltages used here are on the order of transmembrane potentials *in vivo*, indicating that it may be possible to release drugs without any external voltage source by using the weak intrinsic electric potentials of electroactive cells, such as pacemaker cells and neurons.

Release of an anti-cancer drug, MTX

To demonstrate the potential of FePPy NPs in releasing therapeutically relevant molecules, we used methotrexate (MTX) as a model drug. MTX is an anti-cancer drug and controlled delivery through electroresponsive polymers could improve its efficacy. We find that by applying 5 pulses of −0.5 V for 20 s, it is possible to release MTX in a linearly scalable manner. About ~75% of the total drug incorporated could be released (Fig. 5b). This is more than a seven-fold enhancement over drug release from previously reported polypyrrole nanoparticles.[14] We also verified that MTX can be released at ultra-low voltages (−0.075 V, Fig. 5a). It should be noted that MTX is susceptible to electrochemical reduction below −0.6 V at the pH investigated in the study.[24] Therefore, these newly developed NAF are advantageous for the delivery of MTX. Conventional films would require high voltages or longer stimulation times to release the same amount of drug, thereby increasing the chances of drug reduction as well as side reactions.

Biocompatibility of FePPy NPs and bioactivity of released MTX

One concern about using metal based oxidizing agents is the possibility of toxic residue remaining in the polymer backbone which could reduce biocompatibility of polypyrrole.[13] To study the biocompatibility of the NAF, PPy NPs were prepared using $FeCl_3$, without any drug. The NAFs formed were then subjected to 3 stimulations, each consisting of 5 pulses of −0.5 V for 20 s. Control experiments were also performed in the absence of voltage. The electrolyte in contact with the NAFs was then incubated with PC12 cells for 3 days. An MTT assay was performed to assess cell viability. There was no statistically significant difference observed in cell viability when this solution was added to cells, compared to bare cell media, suggesting that the NAFs are biocompatible. Statistical significance between the three groups was determined using the Bonferroni correction to the *p*-value.

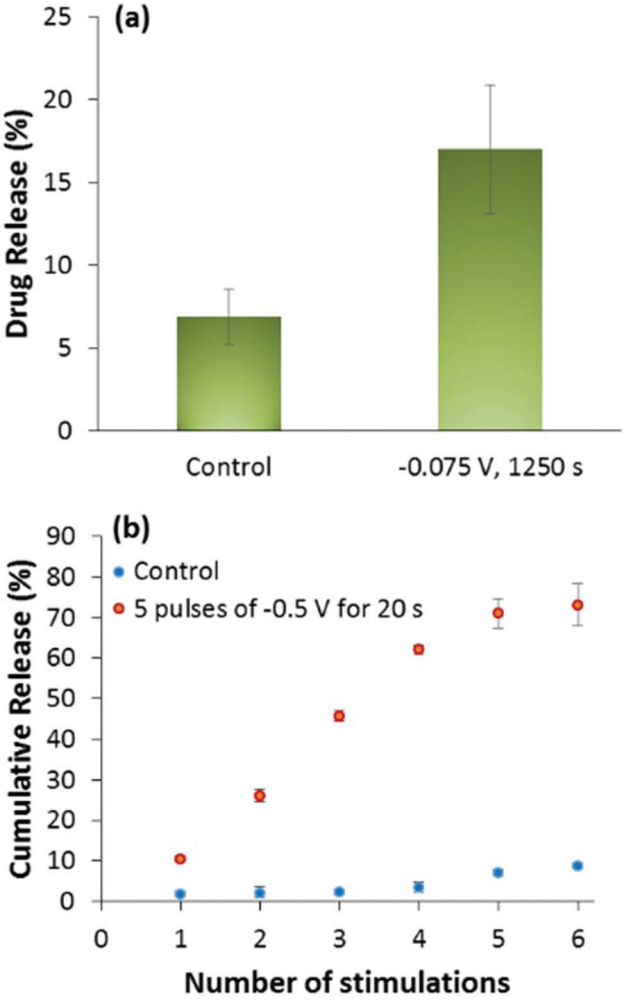

Fig. 5 (a) Ultra-low voltage triggered and (b) pulsed release of MTX.

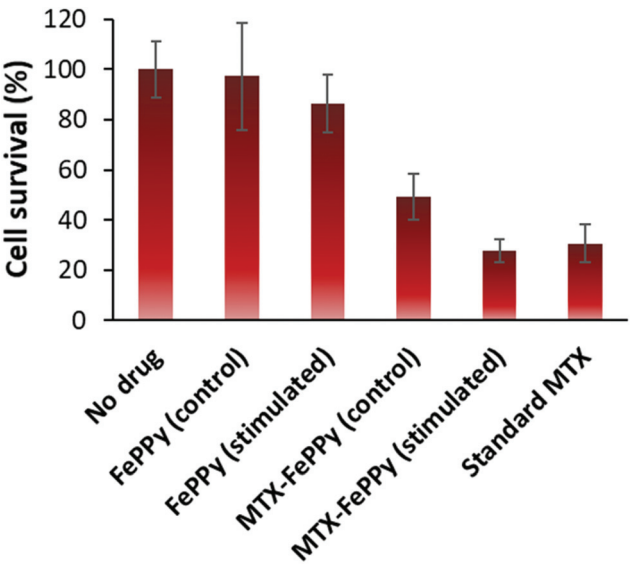

Fig. 6 Biocompatibility of FePPy and bioactivity of released MTX (n = 5).

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To verify the anti-tumor efficacy of the released MTX, a similar procedure was observed in which MTX was released with stimulus. The released solution was added to PC12 cells and the cell viability was measured using an MTT assay after 3 days. As a control, a standard solution of the drug at the same concentration was added to another set of PC12 cells. It can be seen from Fig. 6, that the cell viability is similar for both the released and the standard drug solutions (p = 0.48). These results demonstrate that the bioactivity of released MTX was well-preserved. Some cell death is observed when MTX-containing NAFs are used but no stimulus is applied. This observation corresponds to leakage of MTX from the NAF. In the future, leakage could be addressed by adding a secondary layer of polymer over the NAF.

Conclusions

We have shown that films assembled from polypyrrole nanoparticles have different drug release profiles based on the oxidizing agent used in the synthesis of the nanoparticles. In our studies, the use of metal based oxidizing agents ($FeCl_3$ and $HAuCl_4$) yielded more drug release per stimulus. In addition, when $FeCl_3$ is used, it is possible to release drugs at ultra-low voltages (−0.05 V). We have shown that it is also possible to release MTX, an anti-cancer drug, in a pulsed manner from these NAF at potentials as low as −0.075 V. MTT assay studies reveal that FePPy NPs are biocompatible; in addition, the bioactivity of MTX is preserved after applying the required stimuli. Our results show the importance of the oxidizing agents used in the nanoparticle synthesis on drug release properties. The ability to release drugs at ultra-low voltage signifies that the delivery of redox-active drugs is possible in electrically controlled drug delivery systems, as demonstrated in the case of MTX. Moreover, the lowest voltages that the nanoparticles have been shown to respond to are similar to transmembrane potentials *in vivo*, alluding to the potential of coupling drug release to tissues with intrinsic weak electric potentials.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We are grateful to S. J. Madsen and T. A. Flores for help with TEM and SEM imaging, respectively. DS thanks the Winston Chen Stanford Graduate Fellowship and the Center for Molecular Analysis and Design at Stanford University for funding. ADM is thankful to a Bio-X summer fellowship from Stanford. We are sincerely grateful to C. F. Chamberlayne for technical support.

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