Authors' Accepted Manuscript of:

Vimalarasa, Rubini, Foot, P. J. and Calabrese, Gianpiero (2014) Evaluation of a smart polymer nanosphere for potential use in anticancer drug delivery. Polymers and Polymer Composites, 22(9), pp. 753-762. ISSN (print) 0967-3911

Evaluation of a smart polymer nanosphere for potential use in anticancer drug delivery

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SUMMARY

Polypyrrole-chitosan (ppy-chit) hollow nanospheres have been synthesised using a simple route wherein dispersion polymerization of pyrrole was followed by treatment with aqueous ammonia, centrifugation and dialysis. Ppy-chit hollow nanospheres were characterized by techniques including transmission electron microscopy, atomic force microscopy, powder x-ray diffractometry and UV-visible spectrophotometry. The particle size and stability were assessed by using a Zetasizer. A model anticancer drug, Nile blue chloride, was loaded into the ppy-chit hollow nanospheres by adsorption, and the desorption profile showed that 88% of the dye was released at a typical physiological pH over a period of 5 hours. The combined molecular properties of chitosan and polypyrrole were beneficial to the drug delivery.

Keywords: Core-shell nanoparticles; chitosan; polypyrrole; drug delivery

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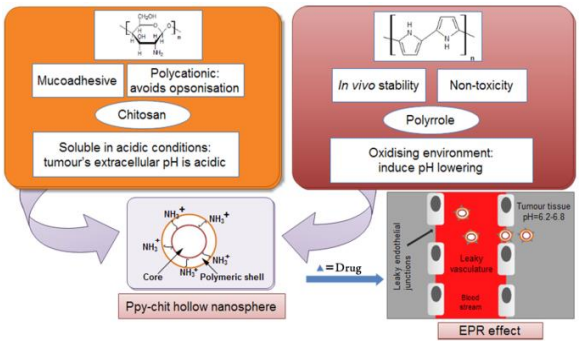
1. INTRODUCTION

Polymer nanoparticles in the range of 1 to 100 nm have been investigated by researchers to treat solid tumours[1]. Their properties present a real advantage for drug delivery, since they can transport an anticancer drug and protect it from premature activation in the blood[2], and clearance from the reticuloendothelial system can be avoided due to their small size. Consequently the drug's toxic effects are reduced and it can be safely delivered into a tumour using the enhanced permeability and retention (EPR) effect[3]. In the present work, core-shell type polymer nanospheres comprising a silver halide core and a polymer composite membrane shell were initially synthesised by modifying a method reported by Cheng *et al* [4], and the AgCl core was then removed chemically. Advantages of this type of nanoparticle for drug delivery include its surface properties which can improve the stealth effect[5] of the core-shell, and the possibility of removing the core to produce hollow nanospheres. Hollow nanospheres can be used as reservoirs wherein a guest molecule such as a drug can become encapsulated within as well as being adsorbed onto the outer surfaces[6].

In our ongoing studies of polymeric hollow nanospheres, we have used biodegradable and biocompatible polymers such as chitosan and polypyrrole for the synthesis of core-shell type structures that could potentially release an anti-cancer drug in a controlled and sustained way[7].

Chitosan (chit), a natural polysaccharide, is a derivative of chitin that is currently used for drug delivery owing to its versatile properties (Scheme 1). Chitosan is polycationic, so it avoids opsonisation, and it is soluble in acidic condition due to the protonation of amino groups. Moreover, the tumour extracellular pH is acidic; hence chitosan nanoparticles can respond to stimuli and release the drug in the microenvironment of the tumour tissue[8]. Polypyrrole is an electrically-conducting polymer that has previously been used in biosensors[9]. Recently it has proved to be promising for drug delivery; Zha *et al* [10] reported that polypyrrole nanoparticles could induce photothermal destruction of cancer cells, due to their strong NIR absorption. In our work, polypyrrole played play a key role in lowering the pH to induce drug release, since it is positively charged when oxidised; therefore in oxygen-rich environments, it can release protons and hence lower the local pH value. This could induce a controlled and sustained release in the appropriate environment. The triggered release of a drug in an appropriate location upon external stimuli such as changes of pH or redox environment would qualify the ppy-chit nanospheres as smart materials[11].



Scheme 1 Schematic of the key properties of hollow polymer NPs for anticancer drug delivery.

This work focuses on chitosan conjugated with polypyrrole to form core-shell nanospheres and studies of their loading with Nile blue chloride as a model anticancer drug. In order to produce ppy-chit hollow nanospheres, two-step pathway was involved wherein dispersion polymerisation[12] was used, followed by an optimized method based on the work of Cheng *et al* for the removal of the inorganic core.

2. EXPERIMENTAL SECTION

All chemical reagents were obtained from Sigma-Aldrich Co.

2.1 Preparation of AgCl- cored polypyrrole-chitosan (AgCl@ppy-chit) core-shell nanospheres

Silver nitrate (0.01 g) and pyrrole (0.025 mL) were carefully added to 20 mL of a chitosan solution (Mw=50-190000, 1wt% in 1 M CH_3COOH). Then iron (III) chloride hexahydrate (0.14 g / 5 mL distilled water) was slowly added dropwise. The reaction mixture was stirred in a chiller at 2°C for 12 hours; then it was purified by dialysis using a membrane bag (Mw cutoff 12-14 000 Da) in 800 mL of CH_3COOH (1 M). A small sample of the purified mixture was diluted and analysed by TEM after

drying on a Formvar substrate. Finally AgCl@ppy-chit core-shells were obtained by precipitating the dialysed solution with acetone and were dried at 40°C for 24 h under vacuum.

2.2 Preparation of polypyrrole-chitosan (ppy-chit) hollow nanospheres

AgCl@ppy-chit core-shell material (0.05 g) was dispersed in water (200 mL) by ultrasonication for 1 hour. The dispersion was then adjusted to pH 11.3 by addition of NH_4OH (5 M) and it was stirred for 24 h. The solution was centrifuged (3500 rpm; 10 min) three times with NH_4OH solution (0.2 M) to remove most of the silver chloride. Any remaining silver ions were removed by dialysis using a membrane bag (Mw cutoff 12-14000 Da) in 3000 mL of NH_4OH solution (0.2 M). Finally ppy-chit hollow nanospheres were obtained by adjusting to pH 4, precipitation with acetone and drying at 40°C for 24 h under vacuum.

2.3 Incorporation of calcein into ppy-chit nanoparticles

Calcein (10 mM, 10% w/v) was added to 20 mL of a chitosan solution (1 wt %, 1M CH_3COOH), and the solution was stirred for 30 min at room temperature before adding pyrrole and Ag NO_3 as above. The procedure was similar to the method reported by Cheng *et al* [4].

AgCl@ppy-chit core-shell material loaded with calcein (0.05 g) was dispersed in water (200 mL) by ultrasonication. The solution was then adjusted to pH 11.3 by addition of NH_4OH (5 M), and was stirred for 24 h. The mixture was centrifuged (3500 rpm; 10 min), three times with NH_4OH solution (0.2 M) to remove most of the silver chloride. Any remaining Ag ions were removed by dialysis using a membrane bag (M_w cutoff 12-14000 Da) in 3000 mL of NH_4OH solution (0.2 M). Finally, ppy-chit nanoshells loaded with calcein were obtained by centrifugation (3500rpm; 10 min) and evaporated on a watch glass, avoiding exposure to light.

2.4 Preparation of Nile blue-loaded ppy-chit hollow nanospheres

Ppy-chit nanospheres previously obtained by the above optimized version of the method reported by Cheng *et al* were adjusted to pH 6.4 with CH_3COOH (1 M). Nile blue chloride in DMSO (10 mM; 10% w/v) was added to the suspension and stirred for 12 h at 250 rpm, avoiding light. The mixture was adjusted to pH 8.5 and was centrifuged (4200 rpm; 90 min). The supernatant was thereby separated from the suspension and the amount of Nile blue chloride encapsulated in the ppy-chit hollow nanospheres was determined by UV-visible spectrophotometry.

2.5 Release study of Nile blue chloride from ppy-chit hollow nanospheres

Ppy-chit loaded with Nile blue chloride as described in section 2.4 (27 mg) was dispersed in phosphate-buffered saline solution (PBS; 2mL) and the dispersion was transferred into a membrane bag (Mw cutoff 12-14000 Da). This was suspended in a 25 mL beaker containing stirring PBS (5mL) solution. The temperature was maintained at 37°C in a water bath to simulate physiological conditions.

A stopwatch was used to monitor the amount of Nile blue chloride released from the ppy-chit hollow nanospheres during different intervals of time. The measurements were carried out by pipetting 2 mL of PBS solution from the reaction beaker directly into a cuvette and the solution was

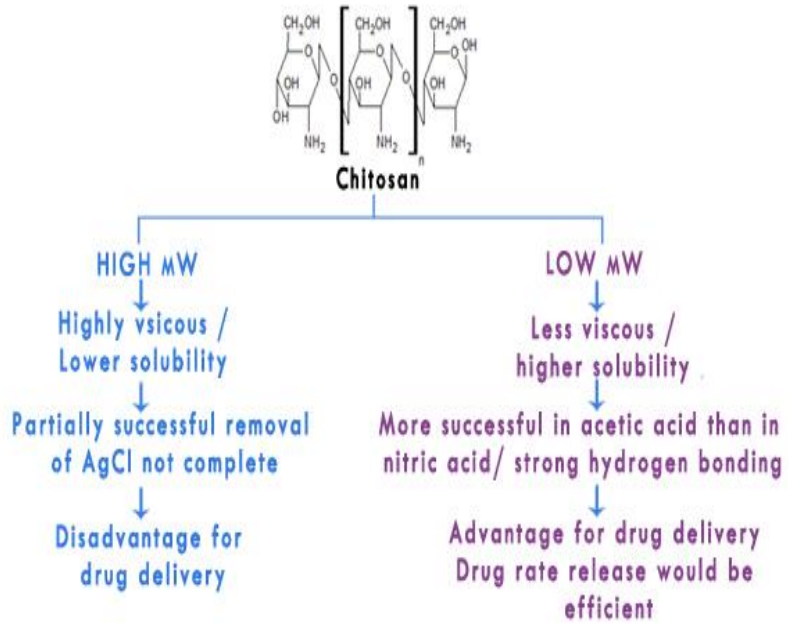
returned to the release medium when the measurement had been completed. Each measurement was made at a wavelength of 651 nm, characteristic of Nile blue chloride in PBS.

Nile blue chloride is sensitive to light; consequently the system was covered by aluminium foil and the beaker was sealed with Parafilm M to prevent evaporation.

3. RESULTS AND DISCUSSION

A modified version of the method reported by Cheng *et al* [4] was used for the synthesis of ppy-chit hollow nanospheres. This method was optimized to suit the purpose of the work which was to load a bioactive molecule into the ppy-chit hollow nanospheres for potential use in anticancer drug delivery.

The procedure described by Cheng *et al* [4] used high molecular weight chitosan, so the polymerization had to be followed by dialysis for the removal of the core AgCl over several days. Application of their method to low molecular weight chitosan in this work was not found to be as successful as for high molecular weight chitosan[13]. The necessary several days of dialysis made the shell of polypyrrole-low molecular weight chitosan fragile, and even then the AgCl core was not completely removed (Figure 2). However, when centrifugation was used, it proved to be efficient for removal of the AgCl (Figure 3), limiting the exposure time of the ppy-chit core shell nanoparticles to ammonia solution and leaving the ppy-chit hollow nanospheres strong and ready for loading with a drug. The core-shell structures were approximately spherical, with a size range of 50-100 nm.



Scheme 2 Summary of the effects of using high and low MW chitosan.

Polypyrrole-chitosan hollow nanospheres were characterized by transmission electron microscopy (TEM), and by other techniques including UV-visible spectrophotometry and X-ray diffractometry.

3.1 TEM Analysis of Ppy-chit with AgCl core, and of the hollow nanospheres

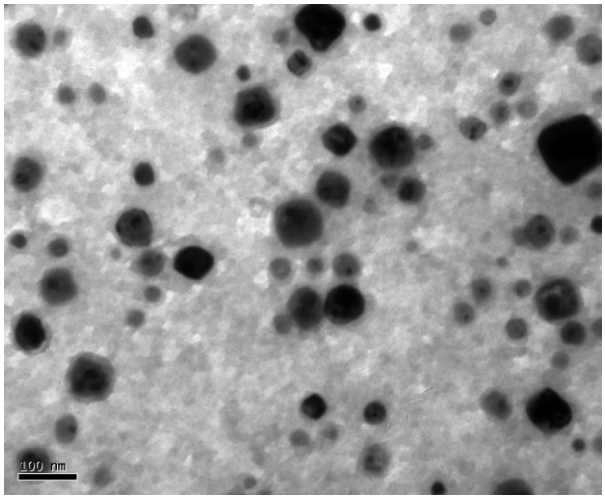


Figure 1 TEM image of AgCl@ppy-chit

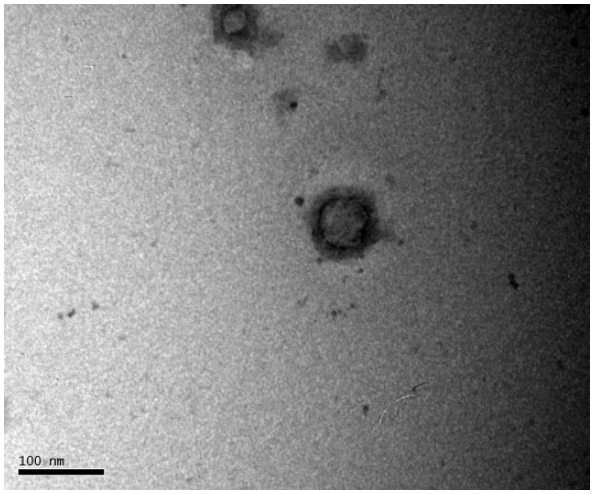


Figure 2 TEM image of Ppy-chit (non-optimized method based on ref. 4)

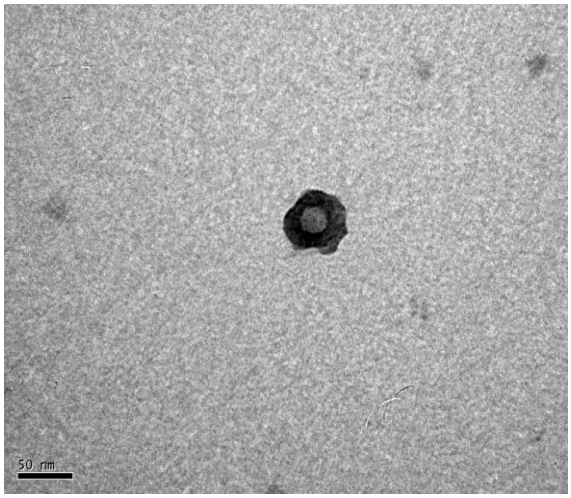


Figure 3 TEM image of Ppy-chit (optimized method)

3.2 UV-Visible and XRD Analysis of Filled and Hollow Core-Shell Nanoparticles

In the UV-visible spectra (Fig. 4) the characteristic peaks for the presence of polypyrrole are at 450 and 300 nm, while the peak characteristic of AgCl is at 250 nm. The absence of the peak at 250 nm indicates that AgCl was removed from the core-shell structure leaving ppy-chit hollow nanospheres.

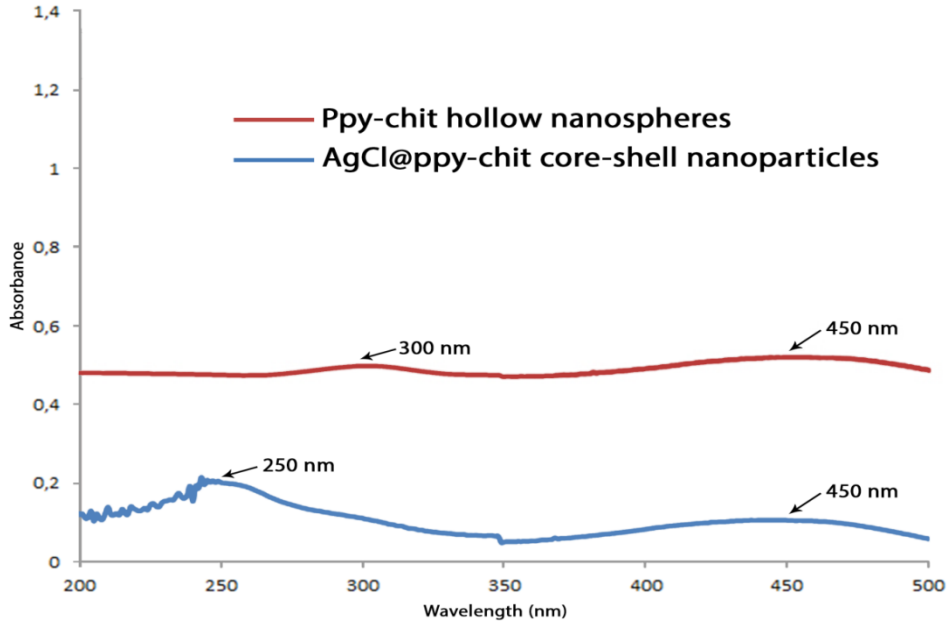


Figure 4 UV-visible spectra of AgCl-filled and hollow nanospheres dispersed in water.

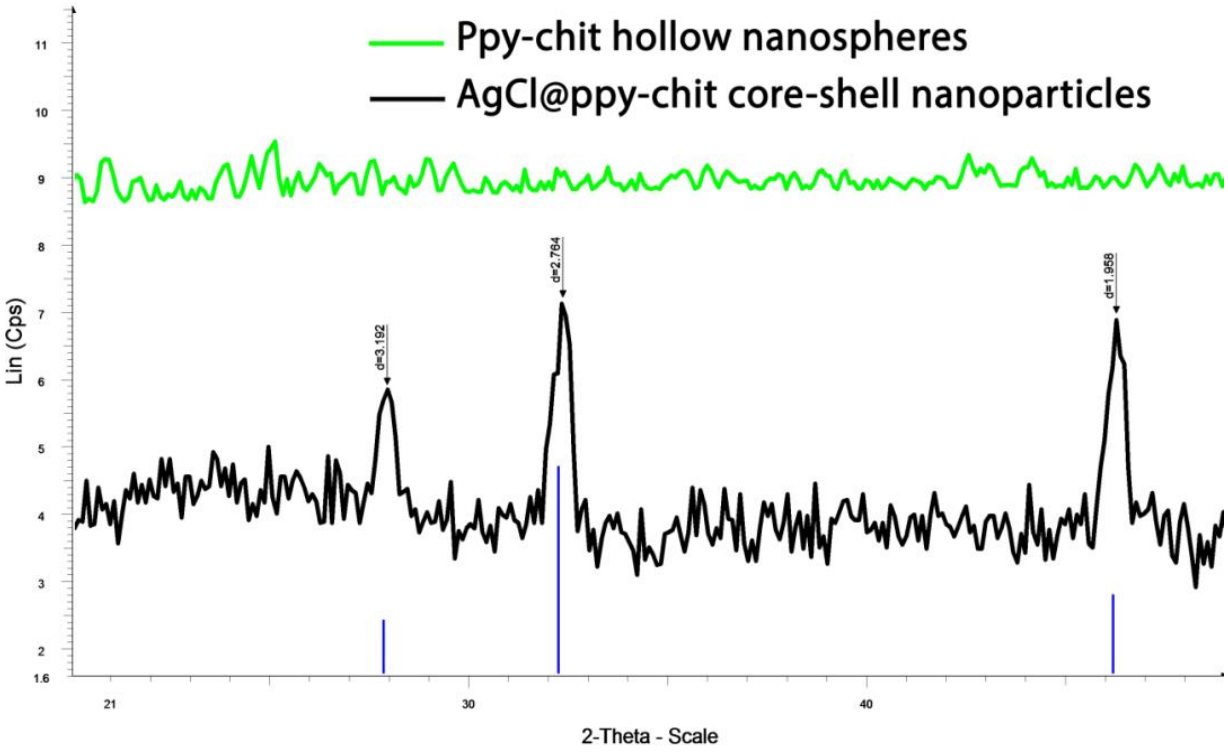
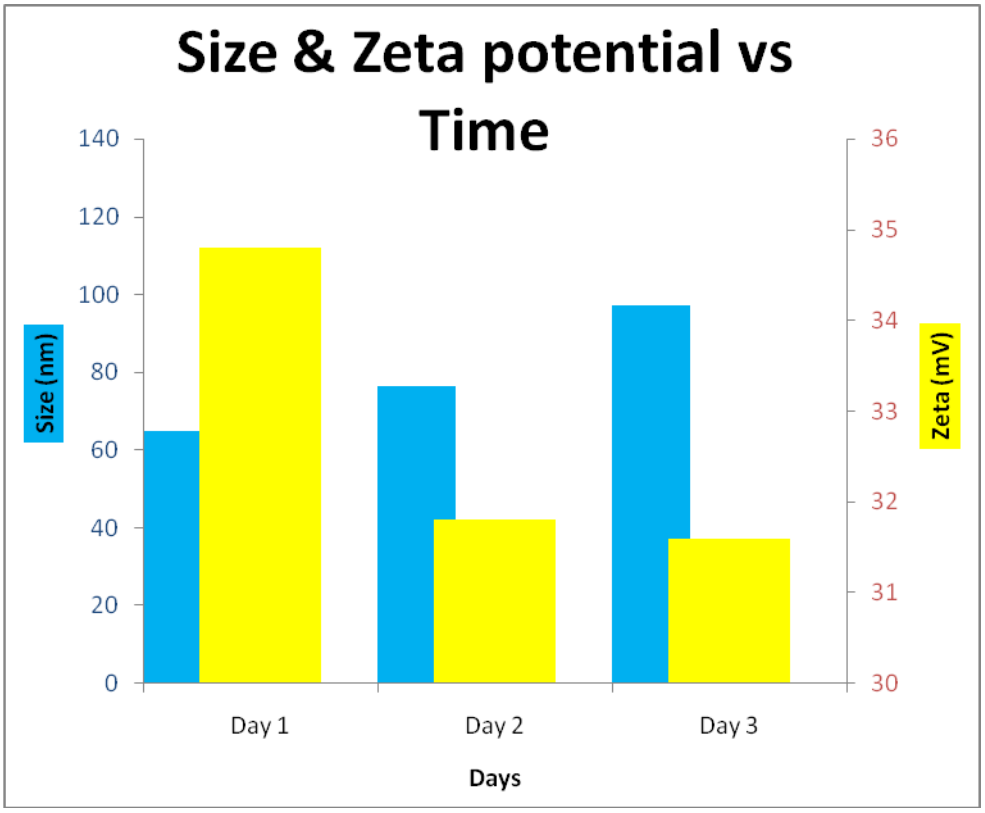


Figure 5 XRD diffractograms of AgCl-filled and hollow nanospheres

In the XRD patterns (Fig. 5) the characteristic peaks for AgCl (argyrite) crystals have d= 3.192, 2.64 and 1.958 A^{o}, and the absence of these peaks confirms that AgCl was successfully removed by the treatment used in this work, leaving hollow nanospheres.

3.3 Measurements of Particle Size and Surface Charge for the Hollow Nanospheres

The size and stability of the hollow nanospheres were investigated using a Malvern Instruments Zetasizer for a period of 3 days under different temperatures at pH 4. Typical data for 23^{o}C are shown in Graph 1.



Graph 1 Particle **s**ize and zeta potential for ppy-chit hollow nanospheres in 10 mM NaCl at 23^{o}C.

The zeta potentials had positive values, mainly due to the cationic tendency of chitosan; at acidic pH values, its amino-groups are protonated, and polypyrrole would have enhanced this tendency, as it also carries a positive charge under most environmental conditions.

These results indicate the likely suitability of the hollow nanospheres to be used for model drug loading. They maintained consistent particle sizes in the range 50-100nm. The zeta potential values of >+30 mV show that the stability of the ppy-chit NPs was high, and they were unaffected by temperatures in the range 20 to 40^{o}C, which would imply stability at body temperature.

The size range of the hollow nanospheres presents an advantage for anticancer drug delivery. Such particles would easily get access to cancer tissues, which have leaky vasculature and capillary pore diameters between 200 and 400 nm. They could hence escape from the reticuloendothelial

clearance system and accumulate any loaded drug via the enhanced penetration and retention (EPR) effect.

3.4 Model Drug Uptake and Characterisation of Loaded Nanoparticles

For the initial studies of the uptake and release of a model drug compound by ppy-chit hollow nanospheres, calcein, a membrane-permeable dye, was encapsulated in the nanoparticles.

The loading was done during the polymerization of the ppy-chit nanoparticles by prior mixing of calcein and chitosan in solution as described in section 2.3. Pyrrole was then polymerized in the presence of oxidant $FeCl_3$ and $AgNO_3$ to form core-shell nanoparticles encapsulated with calcein.

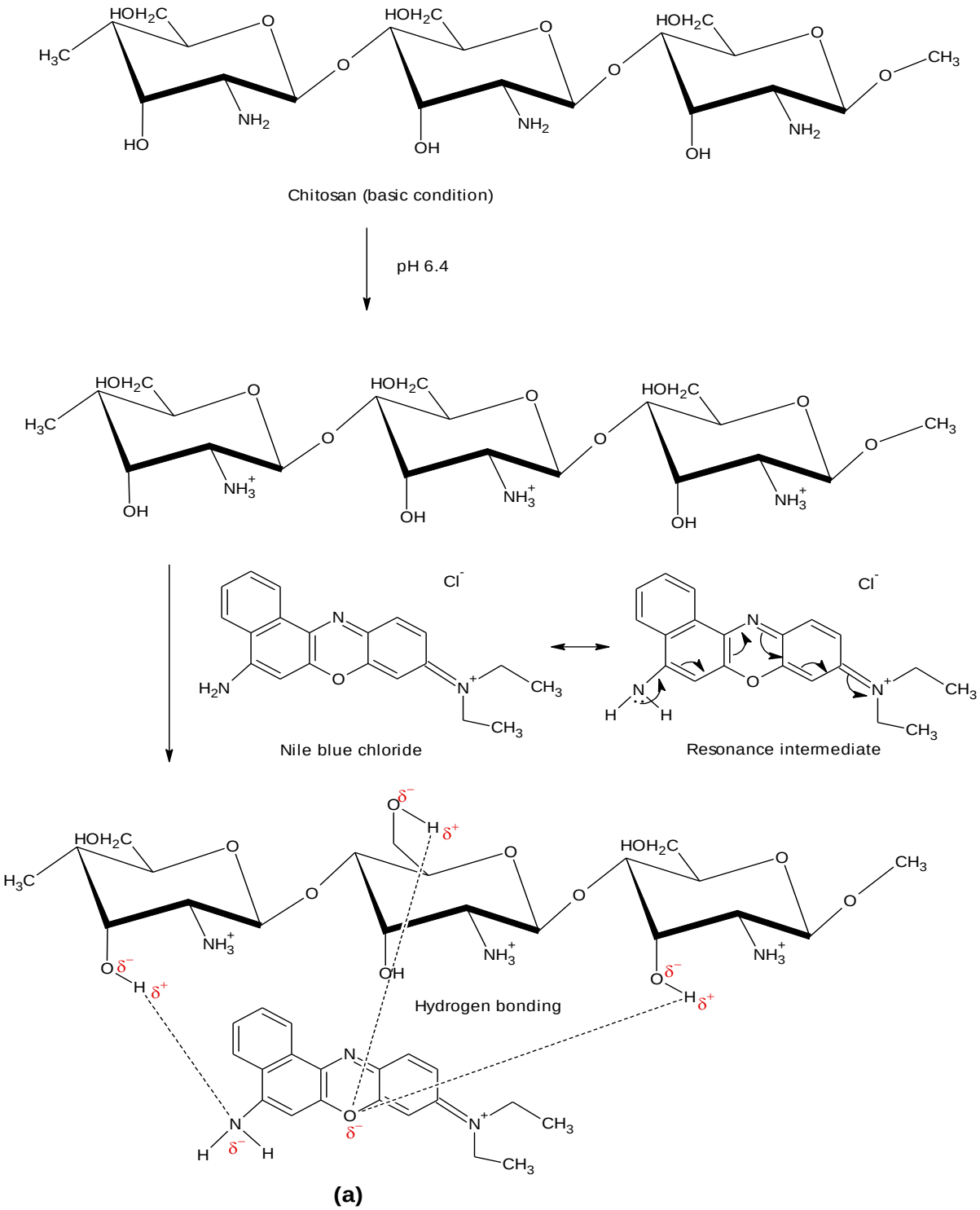
TEM observation showed a full-core structure rather than the core-shell structure seen for AgCl@ppy-chit. This can be explained by the binding of calcein to chitosan which was itself bound to Ag^+ ions via its–OH and $–NH_2$ groups during the formation of a shell around the growing AgCl particles. However, during the removal of the AgCl, the amount of calcein lost was significant, apparently due to its chelation to Ag^+.

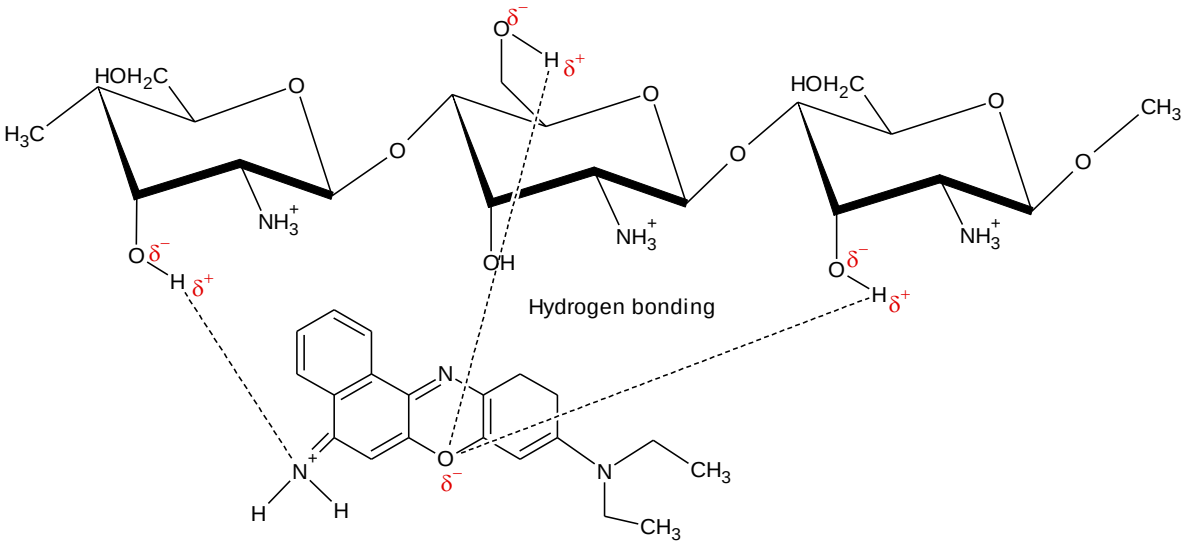
Nile blue chloride was therefore selected as an alternative model compound for the release studies, and it was encapsulated into ppy-chit hollow nanospheres by adsorption (section 2.4). Nile blue chloride is a dye that has itself been used as an anticancer drug; it had advantages for this work such as its flat molecular structure and its presence as a salt, which inhibited any chelation to the Ag^+ ions.

Ppy-chit hollow nanospheres dispersed in ammonia solution were adjusted to pH 6.4. As Cheng *et al* [14] have reported, ppy-chit hollow nanospheres have good permeability for ions, and at pH 6.4, chitosan is at its isoelectronic point. That makes the surface RNH_3^+ concentration minimal, and Nile blue chloride could therefore form hydrogen bonds with free –OH and $–NH_2$ groups of chitosan[15].

Nile blue chloride is a delocalized lipophilic cation, and the proposed forms can be represented by resonance structures after delocalization of lone pairs of electrons present in NH_2 group. The canonical forms of Nile blue chloride are likely to undergo hydrogen bonding with chitosan as shown in Scheme 3, (a) and (b).

Scheme 3 Proposed forms of hydrogen bonding between Nile blue chloride and chitosan





(b)

UV-visible spectrophotometry (λ_{max} = 639 nm) showed that the encapsulation by adsorption was successful, with 88% efficiency under the conditions described in section 2.4. The Nile blue chloride-loaded ppy-chit hollow nanospheres were also characterized by ATR-IR and AFM.

The main characteristic infrared peak for chitosan and polypyrrole is ν_{N-H} stretching at around 3300 cm^{-1}. Its intensity was notably weakened in the nanoparticles, which supports the proposition that there was hydrogen-bonding interaction between chitosan and pyrrole. Peaks observed at 1622 cm^{-1} and 1033 cm^{-1} were characteristic of chitosan's ν_{C-O} stretching, and a peak at 824 cm^{-1} was due to ν_{C-H} bending in polypyrrole.

Peaks characteristic of Nile blue chloride were the ν_{C-O} stretching band at 1641 cm^{-1}, and a ν_{C-C} vibration at 1548 cm^{-1}. Other features were at 1146 cm^{-1} for ν_{C-N} stretching and ν_{C-H} bend at 943 cm^{-1}.

In the ATR-IR spectrum of ppy-chit with Nile blue chloride, the above ν_{N-H} and ν_{O-H} stretching peaks revealed the presence of ppy-chit as they were absent in the spectrum of Nile blue chloride alone. Peaks identifying Nile blue chloride in the loaded ppy-chit sample were a ν_{C-N} stretching band at 1313 cm^{-1} to 1025 cm^{-1} and a ν_{C-C} vibration at 1531 cm^{-1}.

The ATR-IR data were therefore consistent with Nile blue chloride being present in ppy-chit after the encapsulation stage.

3.5 Atomic Force Microscopy (AFM) results for ppy-chit nanospheres before and after dye loading

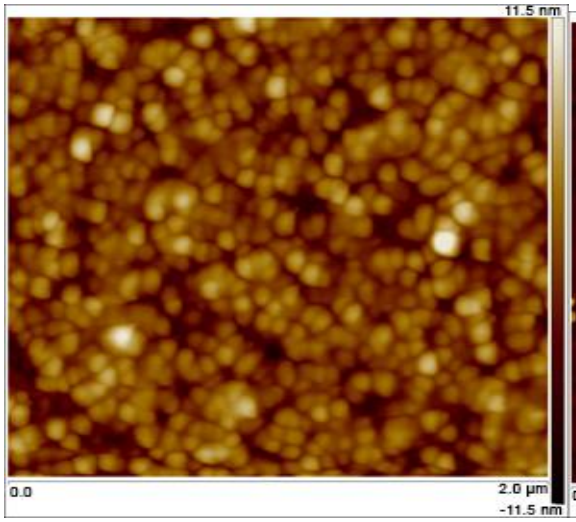


Figure 6 AFM image of AgCl@ppy-chit

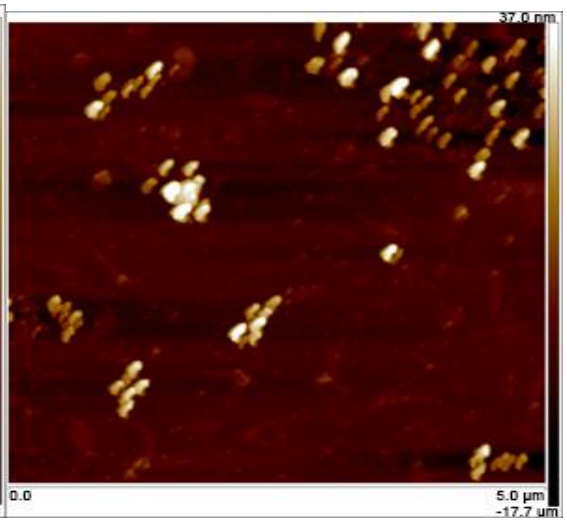


Figure 7 AFM image of Ppy-chit hollow nanoparticles

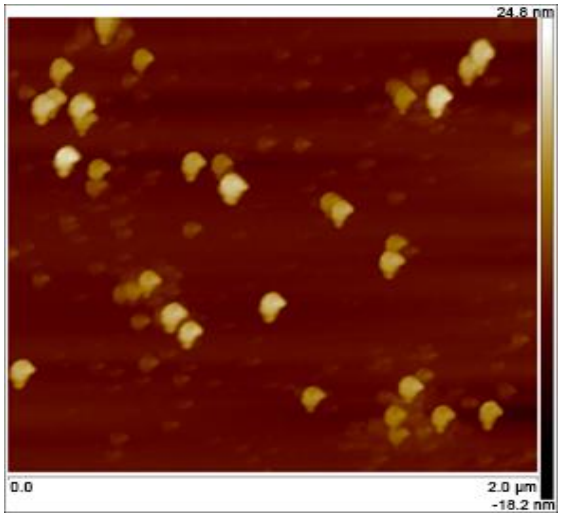


Figure 8 AFM image of Nile blue-loaded ppy-chit nanoparticles

Physical properties	AgCl@ppy-chit	Ppy-chit	Ppy-chit loaded with Nile blue chloride
Height / nm	11 ± 2	10 ± 3	11 ± 2
Diameter / nm	71 ± 9	68 ± 10	62 ± 9
Surface Roughness / nm	2.13	4.19	8.53
Density / μm^{-2}	18.6	4	3.44

Table 1 Physical properties results for nanospheres, obtained from the AFM analysis

The diameter of AgCl@ppy-chit nanospheres was similar to that of ppy-chit hollow nanoparticles; however ppy-chit loaded with the Nile blue chloride was slightly smaller, possibly due to Coulombic attraction to the ionic dye.

The AgCl@ppy-chit nanospheres were found to have a high density due to the presence of heavy atoms in the core. However, after removal of the AgCl, the density was much lower, and the shells had a flatter shape. Nile blue-loaded ppy-chit had a lower density than ppy-chit, indicating that the dye could be partly bound to ppy-chit on the surface rather than exclusively refilling the hollow nanoparticles.

The surface roughness of ppy-chit loaded with Nile blue chloride was significantly higher than that of ppy-chit, which would be consistent with partial coating of the dye onto the outer surfaces of the ppy-chit nanoparticles.

The above data and images give useful information about the morphology of the particles in the nanometre size range[16]. This will help us to understand the encapsulation profile of Nile blue chloride and ppy-chit hollow nanospheres.

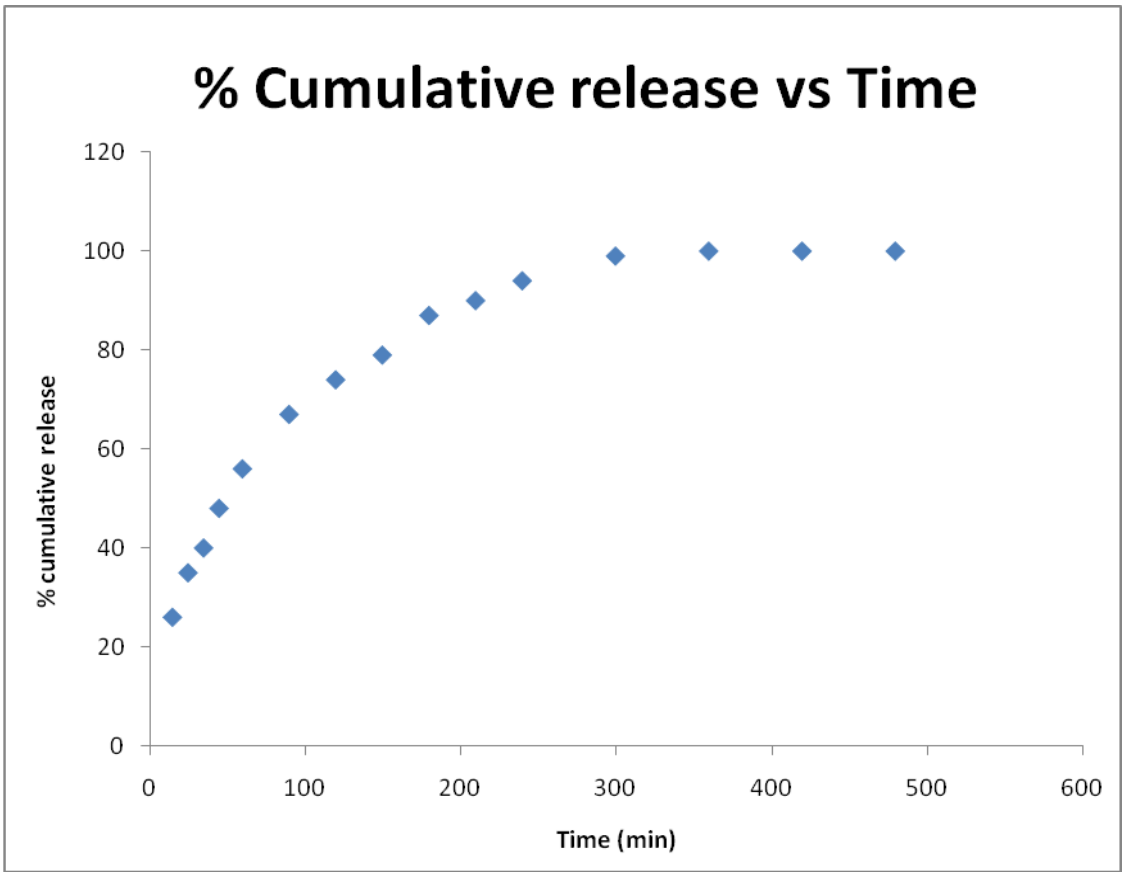
3.6 Model Drug Release Study

A release study was conducted on ppy-chit loaded with Nile blue chloride. The material was suspended in PBS (phosphate buffer saline, pH =7.4). The pH was maintained at pH 7.4, similar to that in the blood, and the temperature was maintained at body temperature, 37◦C.

The physical conditions of the experiment would help to predict the release rate of the dye from the nanospheres in a biological system. At pH 7.4, the amino groups of chitosan were deprotonated, so the nanospheres suspended in PBS had a low solubility in the medium.

The results (shown in Graph 2) reveal an initial burst release of 26% within 15 min, which is probably due to surface-bound Nile blue chloride. The loss of the dye then continued up to 50 % after an hour, and kept increasing for 3 hours. The release rate slowed considerably after 4 hours, becoming zero at the end of 5 hours. After the initial burst, the loss of dye was more consistent with Fickian diffusion of Nile blue chloride from the core of the nanoparticles.

It will be beneficial to conduct a release study at lower pH as chitosan will be protonated below its isoelectronic point (pH 6.4), the dispersibility will be high which will favour controlled release of the dye[17].



Graph 2 Cumulative release of Nile blue chloride as a function of time

4. SUMMARY & CONCLUSION

The method proposed by Cheng *et al* [4] was successfully optimized using low molecular weight chitosan by ammonia treatment followed by centrifugation, avoiding several days of dialysis; this method has proven to be much more convenient in terms of reaction time.

An advantage of using low molar mass chitosan is the relatively narrow size range of the hollow nanospheres obtained, which would be beneficial *in vivo* as 50-100 nm size nanoparticles can escape from the reticuloendothelial system and accumulate any loaded drug in the tumour environment.

The zeta potential of the ppy-chit hollow nanospheres is highly stable at about +30 mV, which means it is positively charged on the surface. The surface charge is important, since the nanospheres loaded with a drug can interact with negatively-charged proteins present on the membrane of the tumour cell, which favours the delivery of the loaded drug[18].

Loading the particles with calcein did not prove to be successful when it was encapsulated during the synthesis of core shell nanoparticles, due to its unfavourable physicochemical properties such as complexation with silver ions. Calcein, being a large molecule, was not very suitable to be loaded by adsorption.

However, Nile blue chloride was chosen to be loading by sorption due to its structure, cationic nature and ability to form hydrogen bonds to chitosan. It also stabilised polypyrrole by preventing it from being reduced at high pH[19]. It was successfully encapsulated with an estimated efficiency of 88 %. Ppy-chit nanoparticles loaded with nile blue were characterized by ATR-IR and AFM.

The characterisation gave strong indications that Nile blue was partially adsorbed on the surface of ppy-chit hollow nanospheres, which is perhaps unsurprising since the average diameter was 62 nm, implying a large available surface area for attachment. As Modica-Napolitano[20] reported, Nile blue chloride is a delocalized lipophilic cation and it has been used as an anticancer agent in the past. Owing to its flat, compact molecular structure it can easily pass through the mitochondria, giving selective cancer cell targeting.

In this preliminary work, the synthesis of ppy-chit hollow nanospheres as a possible drug carrier has been successful. The environmental response of ppy-chit will be further investigated, particularly at low pH and under various redox conditions; it could give a slow, controlled release of therapeutic drugs. Ppy-chit presents a useful combination of properties: polypyrrole could stimulate the release of the drug in an oxidising environment, in where there may be active oxygen species. On the other hand, chitosan itself can respond to pH changes in the microenvironment of the cancer tissue.

ACKNOWLEDGMENTS

We thank Mr Richard Giddens (Kingston University) for valuable TEM support and Dr Dimitrios Lamprou (Strathclyde University) for kindly providing AFM data.

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