The effect of high intensity focussed ultrasound (HIFU) on pH responsive PEGylated micelles

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Over the past two decades much research has been done to discover and optimise the ideal vehicle for targeted drug delivery. One of the largest problems facing drug delivery vehicles is their stability within the bloodstream. The pseudo-peptide poly(L-lysine *iso*-phthalamide) has been investigated as a possible drug delivery vehicle. This particular polymer is biocompatible and biodegradable and in aqueous solution PEGylated versions of the polymer spontaneously forms micelles. The polymer has already been shown to conjugate well with the anti-cancer drug doxorubicin and cellular uptake of the drug-encapsulating micelles has been proven. It has been found that by grafting side chains of amino-poly(ethylene glycol) (PEG) onto the polymer backbone to 40.9 wt%, the resulting polymeric micelles remain stable at very low concentrations and over a large range of pHs, having the potential to be an effective drug delivery vehicle. In this investigation, poly(L-lysine *iso*-phthalamide) was synthesised and grafted with 40.9 wt% PEG in order to determine the limits of its stability between pH 4.0 and 7.4, and at concentrations below 0.88 mg/ml. The micelles were then subjected to high intensity focussed ultrasound (HIFU), and release of encapsulated fluorescent compound Cyanine-5 was observed.

1 Introduction

Most systematically administered drugs are non-target specific, which can lead to unwanted side effects and possible cell damage. A targeted drug delivery system is desirable since a drug could be delivered at its optimum concentration to the affected cells, thereby achieving maximum potential for treatment and minimum damage to healthy cells.

HIFU is appealing for use in targeted drug delivery as it is non-invasive, focused and can penetrate deep within the body [1]. The main mechanisms by which HIFU can be used therapeutically are acoustic streaming (used in low intensity ultrasound), heating and acoustic cavitation [2].

Extensive research has been conducted over the past thirty years to find an ideal, universal drug carrier, key characteristics being: a high drug loading capacity; rapid release possible at target site; stability; biocompatibility; and selective accumulation at the target site.

Micelles made from amphiphlic block copolymers have been investigated as drug carriers for ultrasound enhanced drug delivery, with an important application being within chemotherapy [3, 4]. Due to their hydrophobic interiors, micelles can encapsulate hydrophobic drug molecules enabling them to be solubilised in aqueous environments [5]. PEGylation of the copolymers that form micelles has been used to improve their biocompatibility and stability [6]. Micelles can also accumulate selectively within tumours via the enhanced permeability and retention (EPR) effect [7].

Drugs encapsulated within a micelle, could be subjected to HIFU selectively within the tumour. The ultrasound could both release the drug from the micelle, and also increase poration of the tumour cells (sonoporation) thereby increasing cellular uptake of both micelles and free drug.

We present here an investigation of the stability of PEGylated poly (L-lysine iso-phthalamide) micelles and further explore the effect of HIFU on the release characteristics of the micelles.

2 Materials and Methods

2.1 Materials

L-lysine monohydrochloride (> 99%) was supplied by Lancaster. Isophthaloyl dichloride (98%) and methoxypoly(oxyethylene) amine (mPEO-NH$_2$, amino-PEG, MW 4400) were obtained from Aldrich. Acetone, sodium hydroxide (98.8%) dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were purchased from Fischer Scientific. 4-dimethylaminopyridine (DMAP, 99%) and N,N'-dicyclohexylcarbodiimide (DCC) were supplied by Acros. Cyanine-5 (Cy-5) was synthesised as described by Dai *et al.* [8] and contains two additional carboxylic acid groups and a $CH_3SO_3^-$ species weakly bonded to the nitrogen atom as shown in Fig.1.
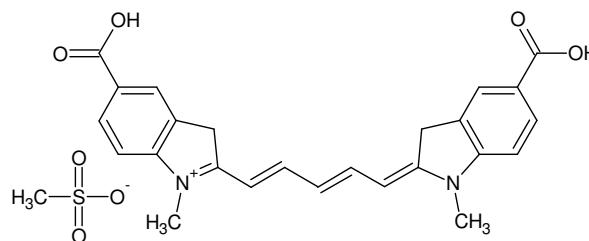

.Fig.1 The fluorescence emission spectra (λ_{ex} = 640 nm) of 0.88 mg/ml micelle solutions (with encapsulated Cy-5) buffered at pH 4.0, 5.0, 6.0 and 7.4.

2.2 Methods

2.2.1 Polymer synthesis

The synthesis of poly(L-lysine *iso*-phthalamide) (PA) and its PEGylated derivatives has been reported previously [5,6,9], with 5% PEGylation of carboxylic acids used.

2.2.2 Cy-5 encapsulation and micelle filtration

PA (0.8 g) and Cy-5 (0.03 g) were dissolved in DMSO (50 ml) and the solution placed inside dialysis membrane (3500 kDa molecular weight cut-off from Medicell International Ltd.). The organic solvent was removed by dialysis against deionised water for 48 h and the volume adjusted to 50 ml with deionised water to give a final

micelle concentration of 0.88 mg/ml. The micelle solution was then filtered through a Millipore Stericup with 0.22 µm pore size to remove any agglomerates or impurities.

2.2.3 Fluorescence spectroscopy

The stability of the micelles was investigated by fluorescence spectroscopy. The emission spectrum of Cy-5 (λ_{ex} = 640 nm) was recorded on a Jobin Yvon Huriba Fluoro Max-3 spectrofluorimeter at right-angle geometry with slit widths of 1 nm, with the emission wavelengths scanned from 650 to 750 nm.

Cy-5 when at high concentration undergoes self quenching, therefore the maximum emission is reduced. The average emission wavelength, λ_{avg}, can be calculated using Eq.(1):

$$\lambda_{avg} = \frac{\Sigma_i \lambda_i I_i}{\Sigma_i I_i} \qquad (1)$$

Where λ_i is the wavelength at which the intensity I_i occurs, and i covers the range of wavelengths under the peak [10]. The average emission wavelength is the intensity-weighted average of the wavelengths scanned, and is a better measure of the shift or change in shape of the emission spectrum than the maximum. It takes into account both the peak shift and the change in intensity, as well as any broadening or skewing of the peak. Broadening or skewing would occur when fluorescence occurs at shifted wavelengths but there are not enough molecules fluorescing at that wavelength to alter the overall maximum of the sample.

Cy-5 is also affected by pH. The unbuffered solution of micelles is at pH 3.9, this acidity is due to the dissociation of the carboxylic acid groups along the polymer backbone. Therefore changes in the maximum fluorescence, and changes in the wavelength at which maximum fluorescence occurs, demonstrate a change in the dilution and pH of the Cy-5 respectively.

2.2.4 HIFU generation

A signal generator (Agilent 33250A) was used to output a 1.1 MHz sine wave, as wave bursts at 5 % duty cycle that were internally triggered with a pulse repetition frequency (PRF) of 1.67 kHz with a 16 s sonication duration. The signal was then amplified with radiofrequency power amplifier (ENI A150) and passed through a matching network with an output resistance of 50 Ω. The HIFU transducer (Sonic Concepts, Model H-102 S/N-8) was measured to have a focal half maximum beam width of 1.2 mm and length of 4 mm with a needle hydrophone (Precision Acoustics SN 1063, 0.075 mm diameter). A 1 ml sample was degassed for 30 mins then placed at the focus of the HIFU transducer within a steel frame sealed with acoustically transparent latex.

2.2.5 Passive cavitation detection

The passive cavitation detector (PCD) transducer (Panametrics Ltd, Model V319-SU) had a centre frequency of 15 MHz, element diameter of 130 mm and a focal length of 760 mm. Inertial cavitation causes the input signal to be redistributed as broadband noise emissions, therefore an increase in amplitude of higher frequencies represents cavitational activity. The high frequencies were detected using the PCD which was confocally aligned with the HIFU

transducer. The PCD output signal was amplified by 40 dB (HP 461A Amplifier), then 5 MHz highpass filtered (Allen Avionics, F5081-5PO-B) to attenuate the 1.1 MHz input signal and lower harmonics allowing measurements of broadband noise. Finally, the waveform was displayed on an oscilloscope (Lecroy Wavesurfer 424).

2.2.6 Measuring inertial cavitation

An increase in the high frequency component of the 'listening' PCD transducer output would result from cavitational activity. The signal processing used was similar to that described by Tu et al. [11]. To process the signal the recorded waveform was converted to the frequency domain using fast Fourier transform (FFT). The amplitude of the FFT between 5 MHz and 15 MHz was calculated for each recorded waveform. The root-mean-square (RMS) of the FFT was determined, giving a graph of RMS values at each time point. By integrating the area under the RMS graphs then dividing by the total HIFU 'on time', the inertial cavitation dose (ICD) could be established.

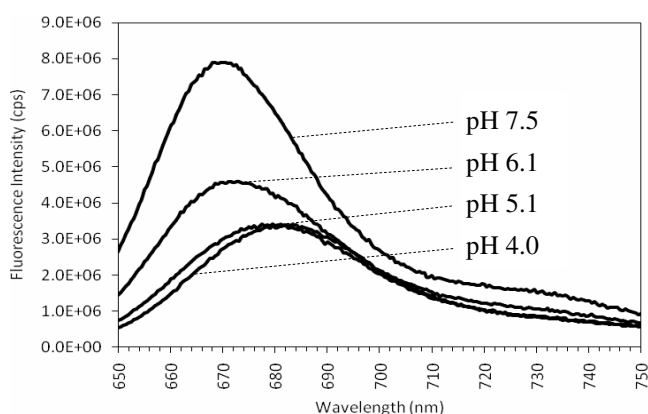## 3   Results and Discussion

3.1   Micelle stability

Fig.2 The fluorescence emission spectra (λ_{ex} = 640 nm) of 0.88 mg/ml micelle solutions (with encapsulated Cy-5) buffered at pH 4.0, 5.1, 6.1 and 7.5.

Fig.2 shows the emission spectra of micelle-Cy-5 solutions buffered at different pHs. Two trends may be observed: firstly that peak wavelength decreases as pH increases, from approximately 683 nm at pH 4.0 to 670 nm at pH 7.4; secondly that fluorescence intensity increases as pH increases, from 3.4 x 10^6 cps at pH 4.0 to 7.9 x 10^6 cps at pH 7.4. Both of these trends suggest the breakdown of micelles as pH increases.

When free in solution, Cy-5 fluoresces maximally at 670 nm. However, when encapsulated within the hydrophobic core of the micelle, the maximum fluorescence occurs at a wavelength of 690 nm. The increase of the peak wavelength of the encapsulated Cy-5 suggests that within the core of the micelle, the Cy-5 is physically interacting with the polymer.

Therefore, a shift in the peak wavelength from 690 nm to 670 nm would suggest that the environment of the Cy-5 is changing from that of the micelle to that of free Cy-5.

Applied to the above data, in pH 4.0 solution at 0.88 mg/ml, the Cy-5 is within the micelle environment hence the majority of micelles are intact. For the same concentration solution at pH 7.4, there is Cy-5 free in solution suggesting deterioration of the micelles.

The increase in fluorescence intensity could be attributed to either: the Cy-5 being released from the core so is no longer self-quenching; or, the fluorescence characteristics of Cy-5 changing between the micelle environment and when it is free in solution. It is clear that within the micelle the Cy-5 interacts with the polymer, this increases the wavelength at which fluorescence is at its maximum.

The change in environment of Cy-5 and the associated peak shift was used as the indicator of micellar breakdown.
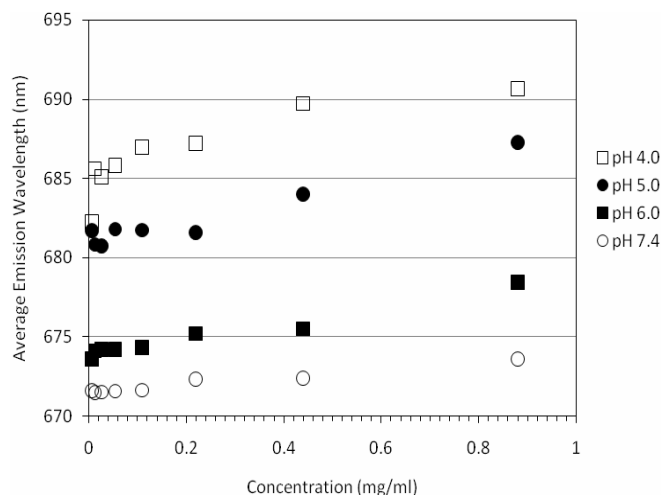

Fig.3 The variation in average emission wavelength (λ_{ex} = 640 nm) with concentration of PA in solutions of pH 4.0, 5.0, 6.0 and 7.4.

Fig.3 shows how λ_{avg} changes with concentration of PA in pH 4.0, 5.0, 6.0 and 7.4 solutions. This plot clearly shows the decrease in λ_{avg} with increasing pH and decreasing concentration, indicating an increasing number of Cy-5 molecules free in solution. The more concentrated samples of micelles in pH 4.0 and 5.0 solution have a very similar λ_{avg} to the 0.88 mg/ml micelle solution, suggesting that the micelles at these concentrations are still intact. The other samples show deviation from λ_{avg} for PA solution, suggesting that micellar breakdown begins instantaneously in buffer solutions for pH 6.0 and 7.4 at all concentrations.
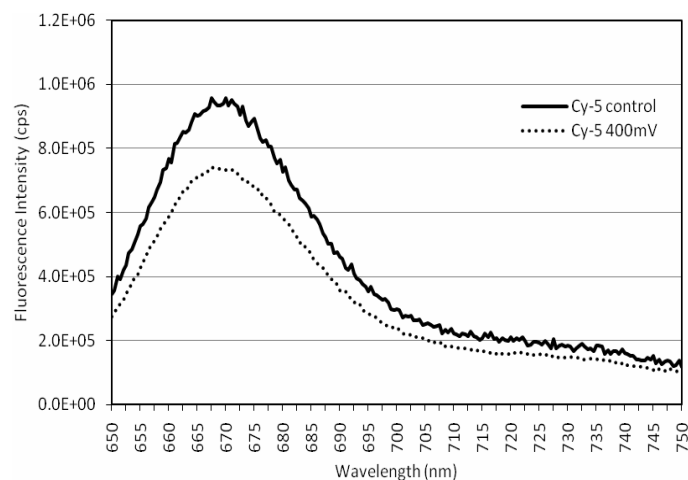
3.2 Ultrasound interaction with Cy-5

Fig.4 The effect of HIFU on the fluorescence of Cy-5 in a pH 5 buffer solution (λ_{ex} = 640 nm). Ultrasound conditions were 2 minutes of 1.1 MHz, 21 MPa peak negative focal pressure, 5 % duty cycle, 1.67 kHz pulse repetition frequency.

Treating Cy-5 with HIFU had an effect on its fluorescence, as shown in Fig.4. This is either due to the disruption of the Cy-5 molecule by the shearing forces [12] or more likely due to the formation of free radicals [13] caused by the cavitating bubbles.

3.3 Ultrasound interaction with micelles

In distilled water the ICD was measured to be 0.049 ± 0.01 whereas with PA micelles the ICD increased to 0.078 ± 0.03. The ICD increase with addition of micelles would be expected as particles in water have been shown to increase cavitation [14,15]. This would indicate an increased likelihood of micellar breakdown due to increased shear forces being applied to the micelles.
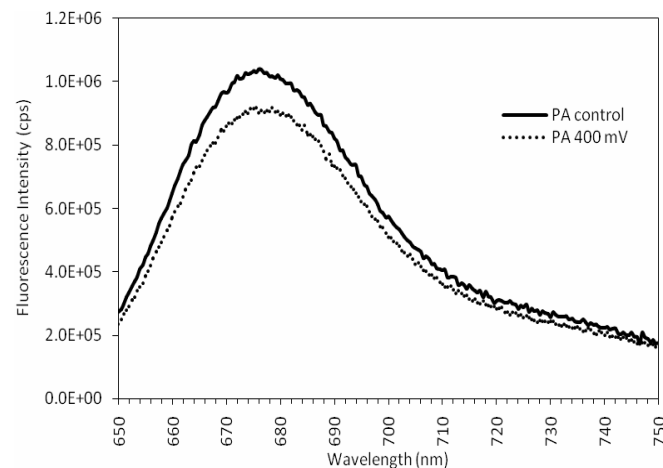

Fig.5 The effect of HIFU on PA micelles in a pH 5 buffer solution. Ultrasound conditions were 2 minutes of 1.1 MHz, 21 MPa peak negative focal pressure, 5 % duty cycle, 1.67 kHz pulse repetition frequency.

Fig.5 shows a decrease in fluorescence with no associated shift in λ_{avg}. This result could be attributed to the dissociation of micelles and subsequent disruption of Cy-5. It is unlikely that the Cy-5 could be disrupted while

remaining within a stable micelle, as the shear forces required to break down the Cy-5 would also destabilise the micelles. It must also be noted that Cy-5 released from the micelles would cause the fluorescence to increase (as quenching would no longer be operating), therefore the overall decrease in fluorescence indicates significant micellar breakdown.

4   Conclusion

The PEGylated micelles investigated appear to be stable at pH 4.0 over a range of concentrations up to 0.88 mg/ml and stable at pH 5 for a concentration of 0.88mg/ml.

The results presented indicate qualitatively that HIFU causes breakdown and release of Cy-5 from PEGylated micelles. Future work should include a more quantitative measure of micellar disruption using a fluorescent compound that would not be affected by HIFU, a possibility being pyrene as it consists of four fused benzene rings.

Acknowledgments

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