Polymersomes and Imaging: A Quantitative Approach

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ABSTRACT

Pericytes are perivascular cells that plays an important role in angiogenesis. To be able to image pericytes can provide useful information on tumour development and treatment response. Here we present the development of an imaging tool for pericyte optical imaging. The tool is based on polymeric manometer-sized vesicles known as polymersomers. We developed a method to quantify polymersomes/cell interactions *in-vitro*. Single cell endocytosis efficiency was calculated combining fluorescence activated cell sorting (FACS) and quantitative reversed phase high pressure liquid chromatography (RP-HPLC) analysis. We show here also preliminary *in-vivo* application of polymersome system for optical imaging.

Keywords: polymersomes, optical imaging, endocytosis

1 INTRODUCTION

To grow and to become metastatic tumours need blood vessels to supply them with nutrients. Angiogenesis describes such situation, where blood vessels are constantly re-organizing and new blood vessels are formed. The formation of new vessels during angiogenesis is regulated by many factors and cells, and among them pericytes. This perivascular cell population is important for the regulation of blood vessels stability: unstable tumour blood vessels presents poor pericytes-endothelial cells association, when compared to healthy blood vessels [1] . Moreover, different tumours presents different pericytes coverage and different response to *stimuli*. Important informations on pericytes role in angiogenesis and response to anti-angiogenic drugs can be achieved through targeted imaging. In this field of research nanocarriers are gaining an increasing attention for both *in-vivo* and *in-vitro* applications [2,3] . Nanocarriers for imaging include quantum dots, fluorescent dyes conjugated with polymers and more complex systems like micelles, liposomes and polymersomes. Among those carriers polymersomes are a promising tool. Polymersomes are nanometre-sized vesicles obtained from amphiphilic copolymers [4] . Because of their vesicular nature they are capable to carry both hydrophilic and hydrophobic probes. Moreover, they can be flexibly engineered to present targeting moieties at the surface. The aim of this work is to develop a polymersome based optical imaging tool to effectively image pericytes. This will be done evaluating *in-vitro* the interaction of polymersomes with relevant cell types, and then moving to the optimization of the system *in-vivo*.

2 EXPERIMENTAL SESSION

2.1 Polymersomes preparation

Copolymers PEO_{113}-$PDPA_{70}$ and $PMPC_{25}$-$PDPA_{70}$ were kindly provided by Prof. Steven Armes Group, Department of Chemistry, Sheffield University, and synthesized as described elsewhere [5] . Copolymers ($20mg$) were dissolved in chloroform/ methanol (2:1) to a concentration of $2mg/mL$ in a glass vial. The solvent was evaporated under vacuum to leave a copolymer film deposited on the walls of the vial. The copolymer film was rehydrated using $2mL$ PBS (100 mm, pH 2). The solution was then sterilized by filtration ($200nm$ pore size). The solution pH was then slowly increased to pH 7.4 using NaOH $0.1M$and the polymersomes dispersion was then sonicated for $45min$. Polymersomes were purified by gel permeation chromatography (GPC) on a size-exclusion column containing Sepharose 4B and PBS (pH 7.4) to elute the polymersomes. Rhodamine labelled formulations were prepared as described above, including 10% molar of rhodamine labelled copolymer PEO_{113}-$PDPA_{70}$ or $PMPC_{25}$-$PDPA_{70}$. Formulations labelled with antibody to PDGFR-β (abcam) were obtained including to the formulations 1% molar of biotinylated $PMPC_{25}$-$PDPA_{70}$. After increasing the pH to 7.4, 1.5 molar excess of streptavidin was added to polymersomes dispersion and stirred at room temperature for $2h$. Dispersion was then added of 1.5 molar excess of biotinylated antibody to PDGFR-β and stirred for $2h$ at room temperature. Polymersomes were purified by GPC as described above. Polymersomes morphology and size were characterised by transmission electron microscopy (TEM) and dynamic light scattering (DLS) respectively. Conjugation with antibody to PDGFR-β was assessed by reversed phase high pressure chromatography (RP-HPLC).

2.2 FACS analysis

Cells were seeded in 24 well plates at a density of $30X10^5$ cells per well. After 24-48h cells were treated with polymersomes (1mg/ml in media). At the desired time-point, cells were washed twice with PBS pH 7.4 Cells were detached with tripsyn/EDTA, pelleted and analysed by FACS (BD FACSArray Bioanalyzer).

2.3 RP-HPLC analysis

Cells were seeded in 24 well plates at a density of $30X10^5$ cells per well. After 24-48h cells were treated with polymeromes (1mg/ml in media). At the desired time-point, cells were washed twice with PBS pH 7.4. All the PBS was removed from the wells and plates were incubated at least 12h at -20℃. Wells were then washed with 200μL of milliQ water acidified with 0.05% of trifluoroacetic acid (TFA). Liquid was transferred to a eppendorf tube and spinned. Surnatant was analysed by RP-HPLC, Dionex Ultimate 3000, λ_{ex}=540nm, λ_{em}=565nm, detector sensitivity 8.

2.4 Endocytosis efficiency

For this studies, and for *in-vivo* studies, we used two different tumour cell lines: mouse fibrosarcoma cells (MFC) expressing different isoform of vascular endothelial growth factor (VEGF): $VEGF_{120}$ and $VEGF_{188}$. These cell lines were used because they originate tumour presenting different pericytes coverage: tumours characterised by $VEGF_{120}$ expression have really poor pericytes coverage when compared to tumours expressing $VEGF_{188}$.

Fitting the data from FACS analysis the time-point at which 100% of cells are positive to rhodamine is extrapolated. Using RP-HPLC data the number of moles of polymers up-taken was calculated and this value was converted into number of polymersomes using the equation 1. Finally, the endocytosis efficiency was calculated using equation 2.

$$N_v = \frac{3 \cdot N_A \cdot g}{4 \cdot \pi \cdot [r + (r - th)]^2} \quad (1)$$

where N_v is the number of polymersomes, N_A is the Avogadro constant, g is the mass in gram measured calculated from RP-HPLC data, r is the radius of the polymersomes measured by DLS and th is the thickness of the polymersome membrane (6nm)

$$EE = \frac{N_v}{N_c \cdot t} \quad (2)$$

where N_v is the number of polymersomes, N_c is the number of cells and t is the time.

2.4.1 *In-vivo* experiments

Dorsal skinflod chamber (DSC) surgery was carried out as described elsewhere [6,7] . Mice were 8 to 12 weeks old. MFC_{188} were grown $1.5X10^5$ cells in 200μL of DMEM media for 72h to obtain spheroid suitable to be implanted in DSC and to originate tumour. After 1 week mice were injected intra-venous (IV) with 100μL of rhodamine labelled polymersomes $\simeq$3mg/mL or $\simeq$1mg/mL. Images were acquired over tumour area with a Zeiss 510LMS equipped with a 10X objective.

3 DISCUSSION

3.1 Poymersomes preparation

In this study we investigated polymersomes as a tool to image a targeted cells population. We adopted a quantitative approach, measuring cellular endocytosis efficiency with respect of two formulations, based on the copolymers PEO_{113}-$PDPA_{70}$ and $PMPC_{25}$-$PDPA_{70}$. Polymersomes were prepared using PEO_{113}-$PDPA_{70}$ or $PMPC_{25}$-$PDPA_{70}$ copolymers, added with 10% molar of rhodamine labelled copolymer. Shape and size were assessed by TEM and DLS respectively (1).

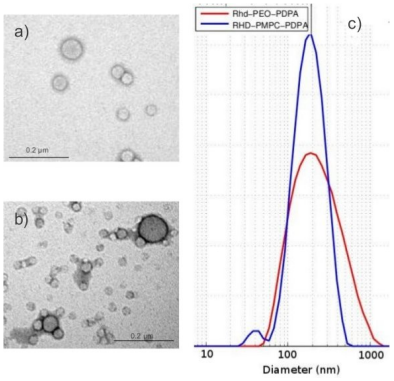


Figure 1: Representative TEM ((a) and (b)) and DLS (c) results. a)PMPC-PDPA polymersomes, b)PEO-PDPA polymersomes. Polymersome membrane stained with phospho tungstenic acid (PTA). Polymersomes have spherical shape, average diameter 200nm

Using the method described above, polymersomes were labelled with antibody to PDGFR-β. After purification, the presence of the antibody in the formulation was assessed by RP-HPLC. The peaks corresponding to copolymer ($PMPC_{25}$-$PDPA_{70}$) and antibody were both individuated (fig.2) in the middle chromatogram (functionalized formulation), while in the control injections of polymersomes alone and antibody to PDGFR-β alone, only one of the peaks can be individuated. The amount of antibody conjugated was 93μg of antibody per 6mg of copolymer.

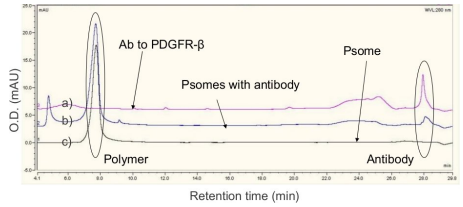


Figure 2: RP-HPLC characterization of functionalized $PMPC_{25}$-$PDPA_{70}$. Bottom chromatogram is pristine polymersomes, middle chromatogram is functionalized $PMPC_{25}$-$PDPA_{70}$, top chromatogram is antibody alone. The first peak (retention time $4.25min$) is streptavidin, the second peak (retention time $7.5min$) is polymer, the last peak (retention time $28min$) is antibody to PDGFR-β.

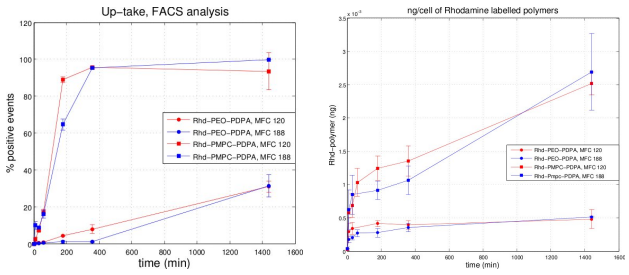
3.2 Endocytosis efficiency

We used two tumour cell lines: MFC_{120} and MFC_{188}. These two cell lines were chosen because they can originate tumour with different pericytes coverage. MFC_{120} tumours are poor in pericytes, when compared to MFC_{188} tumours [8] . Endocytosis efficiency was calculated combining FACS analysis and RP-HPLC analysis, using a method we developed. FACS gave the percentage of cell population uptaking polymersomes. Polymersomes were rhodamine labelled, therefore cells uptaking them became positive to red channel. As can be seen in figure 3 there is a significant difference in the up-take of the two formulations. PEO_{113}-$PDPA_{70}$ is taken-up much slower than $PMPC_{25}$-$PDPA_{70}$ based formulation. In fact, after $24h$ only $\simeq$30% of cells were positive to rhodamine when treated with PEO_{113}-$PDPA_{70}$, when compared to more than 80% of positive cells within $3h$ when $PMPC_{25}$-$PDPA_{70}$ copolymer was used. This suggests that PEO_{113}-$PDPA_{70}$ interacts less with cells. With respect of the cell line, there is no significant difference between MFC_{120} and MFC_{188} , they uptake the two formulation at a similar rate. RP-HPLC analysis confirmed this trend. After extraction of the polymer form cells, we were able to quantify the amount of polymer internalized by cells. Using equation 1 the mass of polymer uptaken was converted in number of polymersomes. As anticipated by FACS results, PEO_{113}-$PDPA_{70}$ based formulation confirmed to be up-taken less efficiently than $PMPC_{25}$-$PDPA_{70}$ formulation (see fig.3. Over $24h$ there is a three fold difference between the two. Again, no significant difference was found between MFC_{120} and MFC_{188} . Using equation 2 we calculated the endocytosis efficiency for both formulations. Results are reported in table 1. Results indicated that spherical nano-sized polymersomes made of PEO_{113}-$PDPA_{70}$ or $PMPC_{25}$-$PDPA_{70}$ have significantly different endocytosis efficiency. PEO_{113}-$PDPA_{70}$ based formulation is up-taken by tumour cells (MFC_{120} and MFC_{188}) less efficiently than $PMPC_{25}$-$PDPA_{70}$ based formulation. This can be explained considering the chemical nature of the two different hydrophilic blocks: PEO and PMPC. They are

Formulation/Cells	EE $(N_v \cdot N_c^{-1} \cdot min^{-1}) \cdot 10^3$
Rhd-PEO-PDPA/MFC120	1.1±0.6
Rhd-PEO-PDPA/MFC188	2.1±0.8
Rhd-PMPC-PDPA/MFC120	248±1
Rhd-PMPC-PDPA/MFC188	132±1

Table 1: Endocytosis efficiency values

both hydrophilic, meaning that they can attract water and create a shield of water around the polymersome that reduce interaction with plasma proteins and cells. This mechanism has been proposed has an explanation of the *stealth* effect of PEGylated liposomes [9] . PEO effect was therefore expected. PMPC is neutral and highly hydrophilic as PEO. However, other works from our laboratory showed strong interaction of the copolymer with cells. This interactions are under investigation. Because we are developing an imaging system to target pericyets, interactions with other cell types, such as MFCs, should be minimized. Therefore, based on this finding, we will focus future work on PEO based copolymer. Moreover, we have functionalized polymersomes with antibody to PDGFR-β to target pericytes. Attachment of antibody to polymersomes has been confirmed by RP-HPLC. Endocytosis efficiency of targeted formulations compared to pristine formulations will be tested, both *in-vitro* and *in-vivo*.



(a) (b)

Figure 3: FACS (a) and RP-HPLC (b) endocytosis efficiency results. FACS results are expressed as percentage of cells positive for rhodamine over time, while from RP-HPLC analysis is possible to calculate the number of polymersomes internalized over time.

3.3 *In-vivo* experiments

Preliminary *in vivo* studies have been performed using pristine PEO_{113}-$PDPA_{70}$ based polymersomes and antibody to PDGFR-β functionalized $PMPC_{25}$-$PDPA_{70}$ polymersomes. Higher concentration ($3mg/mL$) of functionalised $PMPC_{25}$-$PDPA_{70}$ and pristine PEO_{113}-$PDPA_{70}$ were both imaged, while fluorescence signal from less concentrated formulations ($1mg/mL$) were not clearly detected (see fig.4 and 5).

In pictures 4 and 5 a few bright spots can be observed. They could be due to polymersomes accumulating in poorly perfused vessels or to accumulation into

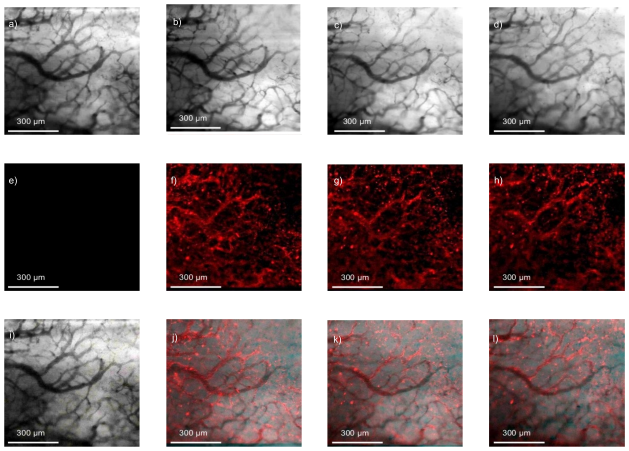


Figure 4: Preliminary in-vivo studies have been performed injecting 100μL of $3mg/mL$ of rhodamine labelled PEO-PDPA polymersomes IV to mice with a fibrosarcoma implanted into a dorsal skinfold chamber. Images were acquired for up to 30min over tumour area using a Zeiss LSM510 confocal microscope. Pictures (a)-(d) transmitted light; (e)-(h) red channel, rhodamine; (i)-(l) merge. Pictures (a),(e),(i), before injection; (b),(f),(j) 1 minute after injection; (c),(g),(k) 21 minutes after injection; (d),(h),(l) 30 minutes after injection.

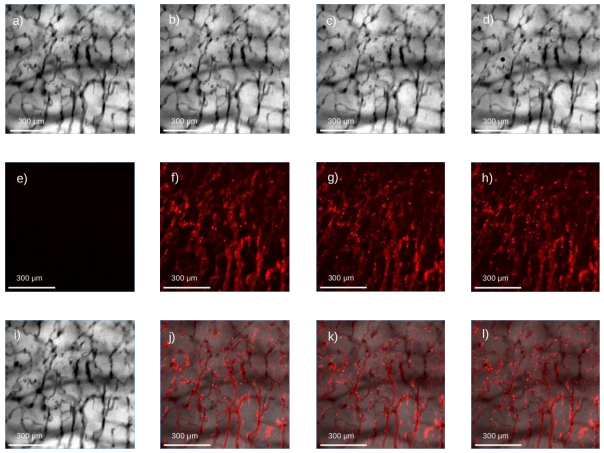


Figure 5: Preliminary in-vivo studies have been performed injecting $100\mu L$ of $3mg/mL$ of rhodamine labelled $PMPC_{25}$-$PDPA_{70}$ polymersomes functionlised with antibody to PDGFR-β. Injection was done IV to mice with a fibrosarcoma implanted into a dorsal skinfold chamber. Images were acquired for up to 30min over tumour area using a Zeiss LSM510 confocal microscope. Pictures (a)-(d) transmitted light; (e)-(h) red channel, rhodamine; (i)-(l) merge. Pictures (a),(e),(i), before injection; (b),(f),(j) 1 minute after injection; (c),(g),(k) 21 minutes after injection; (d),(h),(l) 30 minutes after injection.

lymphocytes. Staining of functional vessels and lymphocytes might clarify the nature of these spots. Furthermore, polymersomes are expecting to extravasate and accumulate around vessels, where pericytes are localised, but with the time-scale ($30min$) of our observation this was not recorded. This could be due to the kinetics of extravasation and accumulation, which could be slower than $30min$. Future experiments will cover longer period of observation. Furthermore, we used here tumour expressing $VEGF_{188}$, which is less haemorrhagic compared to tumours expressing other VEGF isoforms, such as $VEGF_{120}$ [8] .

Overall, we have designed polymersomes functionalized with antibody to target paricytes, we have tested different formulations and calculating endocytosis efficiency we found that PEO_{113}-$PDPA_{70}$ based formulations are up-taken less efficiently compared to $PMPC_{25}$-$PDPA_{70}$ based formulations by MFCs. This provides the rationale to develop PEO based formulations, to target pericytes, in a way that interaction with off-target cells can be minimized. We also performed preliminary *in-vivo* studies, and we can conclude that polymersomes can be used to optically image tumour blood vessels.

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