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**Cellular fluorescein hyperfluorescence is dynamin-dependent and increased by Tetronic 1107 treatment**

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**Highlights**

- Clinical solution-induced corneal staining can be reproduced in an *in vitro* model

- Increased fluorescein uptake does not correlate with necrotic or apoptotic cells
- Dynasore, an inhibitor of dynamin, reduces cellular uptake of fluorescein

- Surfactant Tetronic 1107 increases fluorescein uptake whereas Triton X-100 does not

**Abstract**

Sodium fluorescein ('fluorescein') staining of the ocular surface is frequently an indicator of compromised ocular health, and increases in the presence of certain contact lens multi-purpose solutions (MPS), a phenomenon known as solution induced corneal staining (SICS). The mechanism(s) underpinning fluorescein hyperfluorescence are uncertain, though may reflect increased cellular uptake of fluorescein by corneal epithelial cells. We have developed an *in vitro* model to study fluorescein uptake in both 'generic' mammalian cells (murine fibroblasts) and human corneal cells. Fluorescein hyperfluorescence increased after treatment with two MPS associated with clinical corneal fluorescein staining, yet there was no cellular hyperfluorescence for two MPS that do not cause this staining. Increased fluorescein uptake did not correlate with presence of a necrotic or an apoptotic marker (propidium iodide and Caspase-3 respectively). Incubation of MPS-treated cells with dynasore (an inhibitor of dynamin, implicated in endocytic pathways) reduced fluorescein uptake irrespective of MPS treatment. The non-ionic surfactant Tetronic 1107 (present in both MPS associated with corneal fluorescein staining) increased uptake of fluorescein for both cell types, whereas an unrelated surfactant (Triton X-100) did not. We conclude that the clinical hyperfluorescence profile observed after exposure to four MPS can be reproduced using a simple model of cellular fluorescein uptake, suggesting this is the biological basis for SICS. Fluorescein entry does not correlate with necrosis or apoptosis, but instead involves a dynamin-dependent active process. Moreover the surfactant Tetronic 1107 appears to be a key MPS constituent triggering increased fluorescein entry, and may be the major factor responsible for SICS.

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## 1. Introduction

Sodium fluorescein ('fluorescein') is frequently used in cell biological research, and as a diagnostic tool in ophthalmology and optometry, where it indicates the integrity of the corneal and conjunctival surfaces. When hyperfluorescence (staining) is observed, it is assumed to indicate dead or damaged cells (G. Wilson et al. 1995; Tabery 1992; Tabery 2003a; Tabery 2003b). However, the cellular basis for this staining is uncertain, and may reflect physiological changes unrelated to cellular damage (Bandamwar et al. 2014; Bakkar et al. 2014). Corneal staining is typically observed with pre-existing conditions such as keratoconjunctivitis sicca (dry eye) (Jie et al. 2008; McMonnies 2007), following trauma such as alkali burns or corneal abrasions (Wipperman & Dorsch 2013; Fante & Trobe 2014; S. A. Wilson & Last 2004; Smally et al. 1992; Scarlett & Gee 2007), contact lens wear, and with the use of certain contact lens care solutions (Andrasko & Ryen 2008; Carnt, Willcox, et al. 2007). It remains unclear whether increased fluorescein intensity is localised on the corneal surface, within or between epithelial cells, or within the stroma. It has been suggested that fluorescein can be internalised by epithelial cells (Mokhtarzadeh et al. 2011; Bandamwar et al. 2014) and that it is the increased localisation of fluorescein within the cell that is observed as hyperfluorescence.

Many recent reports have described the phenomenon of solution induced corneal staining (SICS) associated with soft contact lens wear (Efron 2013; Andrasko & Ryen 2008; Carnt, Willcox, et al. 2007; Carnt et al. 2009; Carnt, Jalbert, et al. 2007;

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Morgan & Maldonado-Codina 2009; Ward 2008). SICS presents as superficial corneal and conjunctival staining, consistent with single (or clusters of) cells appearing brighter than adjacent cells (Maldonado-Codina et al. 2013). The intensity is greatest 1-2 hours after exposure to certain soft lens / multipurpose solution (MPS) combinations (Bandamwar et al. 2010; Garofalo et al. 2005; Andrasko & Ryen 2008; Lebow & Schachet 2003). MPS is formulated to both clean and disinfect re-useable contact lenses and characteristically contains a surfactant cleaner (e.g. a poloxamine block copolymer such as Tetronic 1107), a biocide such as Polyhexamethylene biguanide (PHMB) or Polyquaternium-1 (PQ1) and a buffering agent (e.g. citrate, phosphate, or borate).

Certain lens/MPS combinations induce notable cytotoxic effects *in vitro* (Choy et al. 2012; Wright & Mowrey-McKee 2005; Dutot et al. 2008; Dutot et al. 2010), although there is little evidence linking this to fluorescein hyperfluorescence. More broadly it is uncertain whether any cellular damage has occurred when hyperfluorescence is observed *in vivo*. For example, exposure to PHMB-based MPS, most commonly associated with SICS resulted in no histological change when observed using scanning electron microscopy (SEM) (Tchao et al. 2002). Conversely, after exposure to PQ-1-based MPS, typically *not* associated with increased hyperfluorescence, both membrane disruption and increased membrane permeability was observed (Tchao et al. 2002). It has been suggested that SICS is a benign phenomenon which is not indicative of dead or damaged cells (Maldonado-Codina et al. 2013). It is noteworthy that studies of MPS-associated hyperfluorescence predominately focus on the effects of biocides, and there is little

exploration of the potential hyperfluorescence-inducing effects of other MPS components.

An *in vitro* model of SICS potentially provides a useful tool to investigate a) the biological basis of the interaction of epithelial cells with fluorescein and b) a basic understanding of the clinical SICS response. A convincing laboratory model of SICS should exhibit similar hyperfluorescence responses to those observed clinically. Our laboratory has previously developed such models utilising multiple cell types and has shown that fluorescein is taken into cells resulting in hyperfluorescence in response to treatment with a single MPS (Bakkar et al. 2014). We also demonstrated that internalisation and efflux of fluorescein are both energy dependent processes (Bakkar et al. 2014).

The present study aimed to test this model further by confirming that the presence or absence of hyperfluorescence associated with clinical use of four different MPS is also found in our model system. We additionally aimed to clarify the likely mechanisms underlying SICS by investigating whether any hyperfluorescence caused by these solutions is associated with increased apoptosis or endocytosis. There are numerous endocytic pathways that involve dynamin (a GTPase protein) and the actin cytoskeleton. These components in particular are integral to the invagination of the plasma membrane and the movement of endocytic vesicles (that contain extracellular materials) into the cell (Jeng & Welch 2001). Dynamin is a GTPase implicated in several endocytic pathways, and has also been linked to actin cytoskeleton activity (Jeng & Welch 2001; Doherty & McMahon 2009). Both processes are integral to vesicle formation and uptake of extracellular materials into

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the cell body. Dynasore is a well-known inhibitor of dynamin (Tsai et al. 2009; Barrias et al. 2010; Yamada et al. 2009; Macia et al. 2006), and so this was used to investigate dynamin-associated endocytic pathways as a potential mechanism for fluorescein uptake. Finally, we aimed to identify the component(s) of MPS responsible for inducing hyperfluorescence.

## 2. Materials and Methods

### 2.1. Cell culture

Human corneal (HC) cells were obtained from human corneal explants provided by Manchester Eye Bank. Briefly, HC cells were grown from segmented sections of corneal explants in wells pre-coated for 1 hour with 0.01% calf-skin type I collagen (Sigma-Aldrich, UK) prepared in Dulbecco's PBS (PBS) (ThermoFisher, UK) (adapted from Kahn et al. 1993). Murine fibroblasts (L929 cells) (ECAAC, UK) and HC cells were maintained at 37°C and 5% $CO_2$ with Dulbecco's Modified Eagle Medium (DMEM) containing $4500mg^{-L}$ glucose (Sigma-Aldrich, UK) supplemented with 10% v/v fetal bovine serum (FBS) (Sigma-Aldrich, UK) and 4mM L-glutamine (Gibco by Life Technologies, UK), referred to hereafter as 'growth medium'. For HC cells, all tissue culture plasticware had been pre-coated for 1 hour with 0.01% calf-skin type I collagen in PBS. Cells were maintained until they reached approximately 80% confluency, before rinsing with PBS and treating with 0.25% trypsin-EDTA (Life Technologies, UK) for L929 cells or 0.05% trypsin-EDTA in PBS for HC cells, and later neutralisation of trypsin-EDTA with growth medium. For experiments, cells were seeded in 24-well plates (Sigma-Aldrich, UK) at a density of $1x10^5$cell/well for fluorescence microscopy, or in 96 well plates (Sigma-Aldrich, UK) at $2.5x10^4$cells/well for high content analysis (HCA) or cell viability

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experiments, and in CELLview™ glass bottom dishes (Greiner Bio-One, UK) at $5 \times 10^4$ cells per chamber for confocal microscopy.

## 2.2. Cell treatment with MPS, BKC, or MPS components

MPS formulations were obtained commercially, based on their differential association with SICS, and their main biocide and surfactants components (shown in Table 1). MPS known to cause hyperfluorescence were: ReNu Multi-purpose Solution Sensitive® and Biotrue® (Baush + Lomb, Kingston up Thames, UK) referred to as P-1107 and P-PQ1-1107 respectively. MPS investigated which do not cause hyperfluorescence were: Complete RevitaLens® (AMO, High Wycombe, UK) referred to as PQ1-Alex-904, and Opti-Free Replenish® (Alcon, Surrey, UK) referred to as PQ1-Aldox-1304. In later experiments a 10% w/v solution of Tetronic 1107 (BASF, New Jersey, USA) was prepared in borate buffer (0.6% boric acid, 0.1% borax); borate buffer was used as this is the buffering system in use in P-PQ1-1107 and P-1107 formulations, and this was diluted to 1% (the concentration used in those MPS) and also 0.1% in borate buffer prior to addition to cells. A 1.25% solution of Triton X-100 (Sigma-Aldrich, UK) was diluted in PBS to produce 0.125%, 0.0125%, and 0.00125% solutions. Finally 0.01% w/v benzalkonium chloride (BKC) (Sigma-Aldrich, UK) was prepared in PBS. For cell treatment media was removed and the various MPS or surfactant solutions were added, before incubation for 2 hours at 37°C. BKC solution was added for 10 minutes at 37°C (a sufficient time to induce necrosis). Effects were compared with cells treated with growth medium alone, hereafter referred to as 'media control', or another appropriate control.

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### 2.3. Fluorescein staining

After treatment, test formulations were aspirated and cells incubated with 0.01% w/v fluorescein (Sodium fluorescein, Sigma-Aldrich, UK) in PBS for 10 minutes at 37°C. Hoecsht 33342, at a final concentration of 0.2mg/ml in PBS, was added to enable total cell counts (necessary for subsequent fluorescence intensity analysis). For some experiments, propidium iodide (PI) (Sigma-Aldrich, UK), at a final concentration of 0.01mg/ml in PBS, was also added for 10 minutes to identify lysed cells. On removal from the incubator, plates were kept on ice, and cells washed three times with cold PBS; we had previously shown that low temperature was necessary to prevent fluorescein efflux (Bakkar et al. 2014). Cells remained on ice until microscopy or high content analysis (HCA) (always done within 5 minutes).

### 2.4. Metabolic activity assays (MTT)

HC or L929 cells growing in 96 well plates were treated with test formulations, before aspiration and replacement with growth medium containing 0.5mg/ml thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich, UK), and subsequent incubation for 2 hours at 37°C. This medium was aspirated, and insoluble formazan solubilised using 100μl of DMSO (Sigma-Aldrich, UK), followed by assessment of absorbance at 555nm.

### 2.5. Caspase-3 immunofluorescence

L929 cells growing in 24 well plates were treated with MPS and fluorescein as described above. Cells were immunostained with an antibody for cleaved Caspase-3 (New England BioLabs, UK). After fixing for 10 minutes at room temperature in 3.7% w/v paraformaldehyde (Alfa Aesar, UK). A blocking buffer, (5% Normal Goat

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Serum (New England BioLabs, 5425) and 0.3% Triton X-100 (Sigma-Aldrich, UK) in PBS) was then applied for 60 minutes at 37°C. The blocking buffer was aspirated and the primary antibody was diluted 1:400 in 1% BSA (Sigma-Aldrich, UK) supplemented with 0.3% Triton X-100 in PBS (antibody dilution buffer), and applied overnight at 4°C. Plates were washed three times with 1ml PBS and incubating for 5 minutes before the addition of Alex Fluor 594 conjugated goat anti-rabbit IgG secondary antibody (ThermoFisher Scientific, UK) (diluted 1:250 in antibody dilution buffer), and incubated at room temperature in the dark for 2 hours. This was aspirated, and cells washed with 1ml PBS for 5 minutes, before observation using widefield fluorescent microscopy (Olympus IX83).

### 2.6. Evaluation of dynamin-associated fluorescein staining

Cells in 96-well plates were treated with MPS formulations or media for 2 hours and then rinsed with serum free media before addition of 160μM dynasore monohydrate in serum-free medium (dynasore) (Sigma-Aldrich, UK) for 30 minutes. Conditions of dynasore treatment were optimised by fluorescence microscopy analysis of pHRodo™ red transferrin conjugate (Molecular Probes, Life Technologies, UK) uptake by L929 cells (after treatment at 40 to 160uM of dynasore for 30 min to 2hr). The dynasore stock solution contained 0.1% DMSO, and a vehicle control was used in which cells were treated with serum-free medium containing 0.1% DMSO. After aspiration, cells were incubated with 0.01% w/v fluorescein and washed as above. Fluorescein uptake was quantified using HCA.

## 2.7. High content analysis (HCA)

Quantification of fluorescein uptake was carried out using the Cellomics Array Scan VTI HCS Reader and HCS Studio Cell 2.0 Analysis Software. Cells were visualised using the ArrayScan™ automated Observer Z1 inverted microscope (Zeiss) with transmitted light and fluorescence using an XF53 filter wheel. High-resolution images were taken using a CCD camera (Hamamatsu Orca ER) with a 5x/0.25 Fluar objective. Fluorescein stained cell images were taken using 0.033 seconds exposure. One field of view was taken per well, and multiple wells per treatment were tested. Samples were maintained at 4°C during imaging to limit fluorescein loss from cells. Using a built in bioapplication, and adjusting for cell type, the mean pixel intensity of each cell was measured. The median fluorescence intensity value was reported in arbitrary units (AU) (median (lower 95% CI - upper 95% CI limit calculated for the median)).

## 2.8. Fluorescence and Confocal microscopy

Widefield fluorescent images of L929 cells were collected on an Olympus IX83 inverted microscope using a 10x / 0.3 UPlanFL N, or 20x / 0.45 LUC PlanFL N (Ph1) objective and captured using an Orca ER camera (Hamamatsu) with CellSens software (Olympus). Confocal images of L929 and HC were collected on a Leica TCS SP8 AOBS inverted gSTED microscope using a 40x / 1.3 HC PL APO (Oil) objective. The confocal settings were set as pinhole 1.12 airy unit and scan speed 400Hz unidirectional. Images were processed using ImageJ.

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### 2.9. Statistical analysis

All fluorescein fluorescence intensity data are presented as the median intensity values, and error bars represent 95% confidence intervals (CI) calculated for the median. Metabolic activities are presented as a percentage of the appropriate control and the associated CI, calculated using the median metabolic activity values. In all instances, data were analysed using non-parametric Kruskal-Wallis ANOVA and Dunn's multiple comparison post-hoc tests. The level of statistical analysis was set to *p=0.05, **p=0.01 and ***p≤0.001.

### 3. Results

### 3.1. Fluorescein hyperfluorescence *in vitro* is increased by the same MPS formulations associated with SICS.

L929 cells treated with P-PQ1-1107 and P-1107 (MPS formulations both associated with hyperfluorescence clinically) showed increased fluorescein uptake compared to media control, and an increase in PI staining for P-PQ1-1107 only (confirming a lack of cell death for P-1107, and increased late-stage apoptosis or necrosis after treatment with P-PQ1-1107). Those formulations associated clinically with low fluorescein hyperfluorescence (PQ1-Aldox-1304 and PQ1-Alex-904) also caused low fluorescein hyperfluorescence in our model, although increased PI staining was observed for both, suggesting increased late-stage apoptosis or necrosis after treatment with these solutions (Figure 1A).

Fluorescein uptake was quantified using HCA with L929 cells and HC cells, and as its intensity was not normally distributed, median values and the lower 95% CI –

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upper 95% CI of the median were calculated. Intensities after treatment of L929 cells with P-PQ1-1107 and P-1107 were significantly greater than media control (84.5 (78.2-92.3), 119.1 (111.1-126.5) and 61.7 (50.1-71.8) respectively). However for PQ1-Aldox-1304 and PQ1-Alex-904 treatment, intensity was significantly less than media control (10.2 (9.5-10.8) and 0 (0-0) (Figure 1B). Similar results were obtained for HC cells; after P-PQ1-1107 and P-1107 treatment intensity significantly increased when compared to media control (655 (641.3-676.5), 509.9 (492.8-524.6) and 187.5 (180.8-192) respectively) whereas for PQ1-Aldox-1304 and PQ1-Alex-904 intensity decreased (78 (73.2-85.1) and 66 (62.4-71.2) respectively) (Figure 1C). In all instances $p < 0.0001$.

### 3.2. MPS associated with hyperfluorescence cause no change in metabolic activity.

Treatment with cytotoxic levels of BKC resulted in necrosis and minimal fluorescein staining in L929 cells (Figure 2A). MTT assays showed that BKC treated L929 cells retained only 4.4% (4-6.6%) the metabolic activity of control cells ($p = 0.005$). Similarly PQ1-Alex-904 and PQ1-Alex-904+FL treated cells showed 6.4% activity of the control ($p < 0.001$). PQ1-Aldox-1304 and PQ1-Aldox-1304+FL treated cells also showed lower metabolic activity (not statistically different from control). No significant change in metabolic activity was found for fluorescein, P-PQ1-1107 or P-1107 treatment (Figure 2B).

Metabolic activity in HC cells also decreased after BKC treatment to 8.6% (4.5-9.6%) of control; ($p = 0.003$). PQ1-Alex-904 and PQ1-Alex-904+FL treated cells showed decreases to 9.6% (8.9-11.1%) and 4.7% (4.4-7.3%) of control respectively ($p \leq 0.001$).

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PQ1-Aldox-1304 and PQ1-Aldox-1304+FL decreased to 13.7 (11.5-15.1%) and 20.8% (14.1-26.1%) of control respectively (p≤0.03). No significant change in metabolic activity was seen after treatment with fluorescein, P-PQ1-1107 or P-1107 (Figure 2C).

### 3.3. MPS induced fluorescein hyperfluorescence is not associated with apoptosis.

L929 cells and HC cells treated with media control and MPS were examined by confocal microscopy. Membrane blebbing (a hallmark of apoptosis) was observed for P-PQ1-1107 treatment but not after media control or P-1107 treatment. Since both these MPS are associated with hyperfluorescence, these data suggest apoptosis and hyperfluorescence may be distinct processes (Figure 3A). To examine further, L929 cells treated with various MPS formulations and fluorescein were examined for apoptosis by immunostaining for cleaved caspase-3. With all MPS treatments, no clear evidence of caspase-3 staining above media control levels was apparent (Figure 3B), indicating no correlation between hyperfluorescence and apoptosis.

### 3.4. Fluorescein staining in L929 and HC cells is associated with dynamin-dependent pathways.

Dynasore treatment resulted in decreased fluorescein intensity in control L929 cells and after treatment with hyperfluorescence-inducing MPS formulations (p<0.0001 in all instances) (Figure 4A). The same effect was also seen in HC cells, with a significant decrease in dynasore treated cells (Figure 4B) (p≤0.002). No significant difference in cell numbers was found after treatment with or without dynasore for both control or MPS-treated cells. Additionally, metabolic activity measured for cells treated under identical conditions was measured by MTT assay. No significant

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decrease was found (Figure 4C and 4D respectively), confirming that the decreased fluorescein intensity seen after dynasore treatment was not simply an artefact related to a toxic effect of dynasore itself.

### 3.5. Increased fluorescein fluorescence is related to the surfactant Tetronic 1107.

The two solutions causing increased fluorescein hyperfluorescence both contain Tetronic 1107, and so we tested whether this might cause hyperfluorescence, both above and below its critical micelle concentration (CMC) (CMC=0.49% w/v at pH 7.4 (Chiappetta et al. 2008)).  Cells were treated with Tetronic 1107 at 0.1%, 1% and 10% w/v for 2 hours before fluorescein treatment. Fluorescein fluorescence intensity in L929 cells increased with 0.1%, 1% and 10% w/v Tetronic 1107 compared to both borate buffer (carrier control) and media control (p<0.0001 in all instances) (Figure 5A). There was a small but significant increase in fluorescence intensity after 1% Tetronic 1107 treatment to 642.5 (632.5-653.3) compared to cells treated with P-PQ1-1107 at 604.5 (584-621.4); p<0.0001). As expected the median fluorescence intensity decreased for PQ1-Aldox-1304 and PQ1-Alex-904 treatments relative to both buffer and media control (p<0.0001).

In HC cells, 1% w/v Tetronic 1107 treatment significantly increased the median fluorescein fluorescence intensity to 227.3 (215.2-238.8) as compared with borate buffer treatment and media control (p<0.0001 in both instances). The fluorescence intensity found for 1% w/v Tetronic 1107 also significantly exceeded the intensity measured for PQ1-Aldox-1304, PQ1-Alex-904, 0.1% w/v and 10% w/v Tetronic

1107, however were less than the median fluorescence intensity values found for P-PQ1-1107 and P-1107 (p<0.0001 in all instances) (Figure 5B).

The effect on fluorescein fluorescence of treatment with the generic surfactant Triton X-100 was also measured in HC cells (Figure 5C). The CMC for Triton X-100 occurs between 0.125 to 0.563% (v/v). Fluorescein fluorescence intensities after 1.25%, 0.125% and 0.0125% Triton X-100 treatment were significantly less than after PBS treatment of HC cells (p<0.0001 in all instances). The fluorescence intensity after 0.00125% Triton X-100 treatment of HC cells was comparable to that of PBS treated cells (p>0.9999). Treatment with 0.00125% and 0.0125% Triton X-100 significantly increased fluorescein fluorescence intensity to 307.8 (298-313.5) and 244.6 (240.8-249.6) respectively relative to media control (212.8 (207.9-216.5)) whereas after treatment with 0.125% and 1.25% Triton X-100 (concentrations *above* its CMC) a decrease in fluorescein fluorescence intensity to 32.5 (31.2-34.8) and 22.6 (22.4-22.8) respectively was observed (p<0.0001 in all instances).

## 3.6. Surfactant-induced hyperfluorescence is not mediated by increased metabolic activity.

The effects of Tetronic 1107 and Triton X-100 on cell metabolic activity was examined to confirm that surfactant-induced hyperfluorescence was not mediated through increased cellular activity. We found that 0.1%, 1%, and 10% w/v Tetronic 1107 treatments resulted in no significant change in metabolic activity for L929 cells (p>0.9999 in all instances) and for HC cells (p≥0.6) (Figures 5D and E respectively).

The effect of Triton X-100 on HC cell metabolic activity was also measured. No significant change in metabolic activity was observed with fluorescein only, PBS+FL,

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0.00125% and 0.0125% Triton X-100+FL treatments (p>0.999 in all instances). However, with 0.125% and 1.25% Triton X-100 treatment metabolic activity significantly decreased to 1.9% (1.7-2%) and 1.5 (1.2-2%) respectively; (p=0.01 and p=0.003 respectively) (Figure 5F).

## 4. Discussion

We have developed an *in vitro* model to investigate the biological basis for corneal fluorescein hyperfluorescence. Our approach made use of the well-characterised clinical effects of different contact lens MPS on fluorescein hyperfluorescence (known as SICS). Fluorescein hyperfluorescence may involve the direct uptake of fluorescein into corneal epithelial cells (Mokhtarzadeh et al. 2011; Kuwayama et al. 2002; Bandamwar et al. 2014). We previously showed that fluorescein uptake by cells was temperature dependent, and increased after exposure to ReNu Multiplus® MPS, which contains Tetronic 1107 like P-1107 (Bakkar et al. 2014). In the present study, we examined the effects of MPS known to increase or decrease hyperfluorescence clinically, and further examined the biological basis for fluorescein uptake. We tested this after a 2 hour exposure to MPS, which clinically is the exposure time typically found to produce maximal corneal staining, and which is the exposure time used in the Andrasko Staining Grid (Andrasko & Ryen 2008).

Notably we performed fluorescein quantification using a high content analysis (HCA) platform which measures fluorescence levels in each cell. MPS formulations associated with increased hyperfluorescence on the ocular surface (P-PQ1-1107 and P-1107) (Jones et al. 2002; Andrasko & Ryen 2008; Tilia et al. 2012) both cause

increased fluorescein hyperfluorescence in cells. Conversely PQ1-Alex-904 or PQ1-Aldox-1304 resulted in little or no fluorescein staining, also as is found clinically (Kilvington et al. 2010; Tilia et al. 2012). The interaction of a contact lens material itself with the MPS may contribute to the hyperfluorescence observed clinically. However our finding that the clinically observed effects of these four MPS can be reproduced without the presence of a lens, suggests that SICS may primarily involve the biochemical effects of the MPS alone.

Non-viable necrotic cells (following BKC treatment) are unable to take up fluorescein, consistent with our previous findings (Bakkar et al. 2014) and with those of Bandamwar *et al* who reported minimal fluorescein fluorescence in non-viable human corneal limbal epithelial (HCLE) cells (Bandamwar et al. 2014). Interestingly MPS formulations P-PQ1-1107 and P-1107, both of which are associated with clinical hyperfluorescence, produced limited or no increased PI staining respectively and no significant decreases in metabolic activity. Conversely, significant loss in metabolic activity was found with the MPS formulations PQ1-Alex-904 and PQ1-Aldox-1304, both of which are clinically associated with low levels of fluorescence. In contrast with the view that fluorescein hyperfluorescence reflects 'toxicity staining', fluorescein staining appears in fact to associate with an absence of cytotoxicity. Staining on the ocular surface may therefore not indicate dead cells, but instead metabolically fully functional cells. The presence of MPS increases fluorescein staining in these living cells. In the later stages of our study we examined what processes - presumably stimulated by MPS P-PQ1-1107 and P-1107 – may be responsible for this hyperfluorescence.

Notably we did not find clear evidence for any of the test MPS formulations resulting in increased staining for Caspase-3, which is a vital downstream protease in the apoptosis pathway (Li & Yuan 2008; McIlwain et al. 2013), suggesting hyperfluorescence does not correlate with apoptosis. Interestingly, blebbing (a morphological change of the cell membrane associated with apoptosis (Mills et al. 1998; Ziegler & Groscurth 2004)) occurred in P-PQ1-1107 treated cells, although not after P-1107 treatment, even though both increase hyperfluorescence *in vitro* and *in vivo*, further suggesting apoptosis and hyperfluorescence are distinct processes. The lack of increased Caspase-3 staining after P-PQ1-1107-treatment suggests the apparent apoptosis indicated by blebbing in P-PQ1-1107-treated cells has progressed beyond the early-stages at the time of assay (further work with specific markers for late-stage apoptosis would confirm this).

We previously reported that fluorescein cellular uptake and egress are active processes (Bakkar et al. 2014). We investigated this further here by directly inhibiting dynamin-dependent pathways using dynasore, a reversible inhibitor of dynamin, and found decreased fluorescein uptake for both control and MPS-treated cells. These data suggest dynamin and its associated actin binding proteins are directly involved in fluorescein cellular entry. However, as fluorescein uptake was not completely inhibited by dynasore, it is possibly other endocytic pathways independent of dynamin may also contribute.

We finally examined which MPS components may increase the active internalisation of fluorescein. The non-ionic surfactant Tetronic 1107 is present in both P-PQ1-1107 and P-1107 (the MPS which increase fluorescein staining) and has the lowest

ethylene oxide to propylene oxide ratio and is thus highly hydrophilic, in contrast to the less hydrophilic surfactants Tetronics 904 and 1304, which are present in PQ1-Alex-904 and PQ1-Aldox-1304 respectively (Table 2) (Alvarez-Lorenzo et al. 2010).

Our finding that hyperfluorescence intensity increases significantly after treatment with 1% (w/v) Tetronic 1107 (the concentration in P-PQ1-1107 and P-1107) suggest Tetronic 1107 is a key mediator of hyperfluorescence caused by these MPS. Tetronic 1107 forms polymeric micelles which are more stable and less damaging to the lipid bilayer than typical surfactant micelles (Sandez-Macho et al. 2015). Polymeric micelles encapsulate and solubilise otherwise poorly water-soluble drugs, and block copolymers have drug delivery properties (Alakhov et al. 1996; Harada & Kataoka 1998; Cammas et al. 1997). Polymeric micelle uptake is time-, pH-, energy-, and temperature-dependent (Allen et al. 1999; Luo et al. 2002), with localisation to cytoplasmic organelles (Savic et al. 2003), and may be mediated by endocytosis (Allen et al. 1999; Luo et al. 2002; Savic et al. 2003). Our finding of a role for Tetronic 1107 in increasing fluorescein hyperfluorescence, is therefore consistent with our identification of dynamin-dependent processes (which include endocytosis) as a key mediator of MPS-induced fluorescein hyperfluorescence.

In contrast, we found the surfactant Triton X-100 does not induce increased fluorescein uptake in HC cells when used at levels above its CMC, suggesting Tetronic 1107 induced hyperfluorescence may be specific to this block copolymer, and cannot be replicated with a similarly non-ionic surfactant. Triton X-100 causes cell lysis (Koley & Bard 2010), consistent with our finding of a significant decrease in metabolic activity after treatment. This may counteract any increase in

hyperfluorescence (in line with our previous findings that non-viable cells exhibit minimal hyperfluorescence).

Our study is the first to investigate the effects of surfactants on fluorescein uptake, and suggests that hyperfluorescence could be attributed to the surfactant component in MPS formulations. Although interactions of the other MPS components in these solutions and the presence of a contact lens may contribute to the overall hyperfluorescence, our data implicate Tetronic 1107 effects on active uptake processes in the cells as being central to SICS. More broadly our findings suggest that active dynamin-dependent processes may be a key component of corneal fluorescein hyperfluorescence caused by a variety of stimuli.

### Acknowledgements

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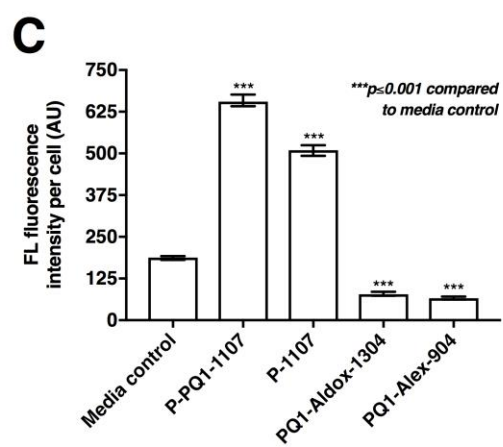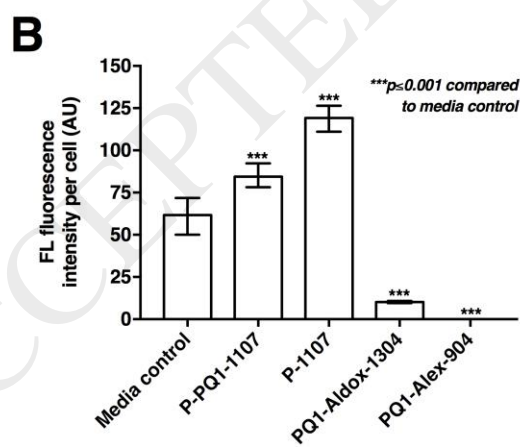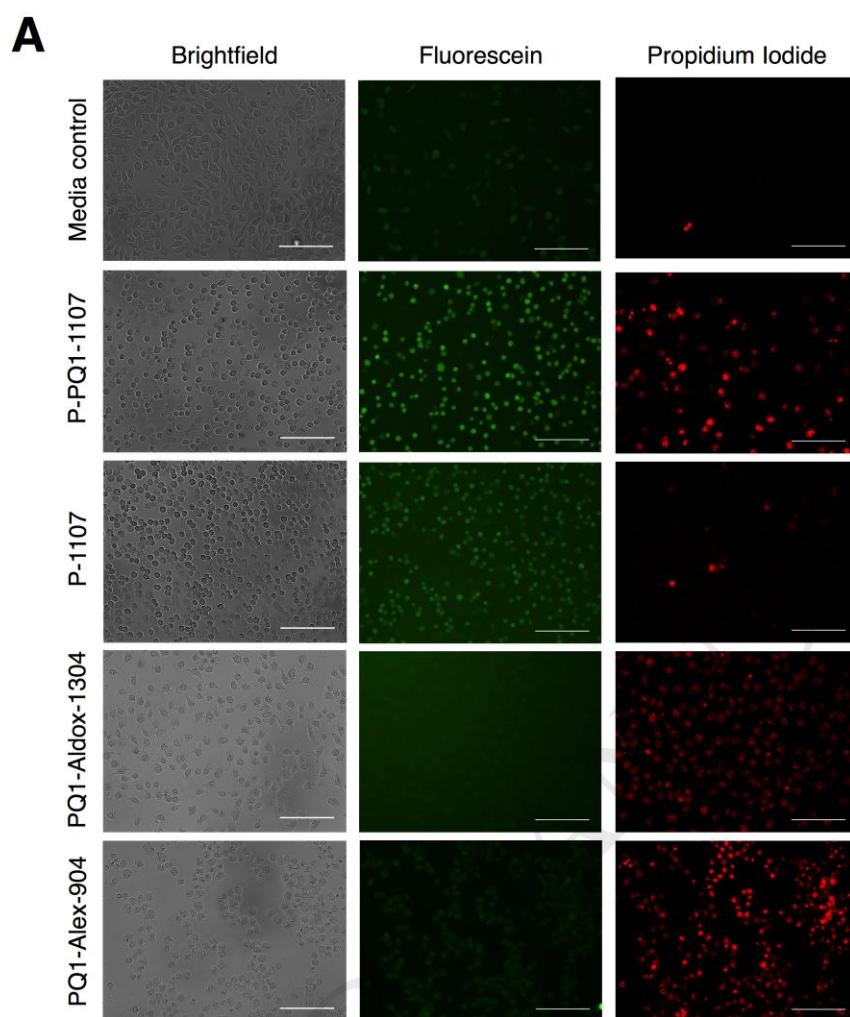
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**Figure 1**: **Fluorescein and propidium iodide staining after MPS treatment.** L929 cells were treated with MPS, and then stained with 0.01% w/v fluorescein or propidium iodide. **(A)** Representative fluorescence microscopy images show differential intensities of fluroescein staining with MPS treatment in L929 cells. **(B)** Quantification of fluorescence intensity in MPS-treated L929 cells or (C) MPS-treated HC cells by HCA using the Cellomics Array Scan VTI HCS Reader. Fluorescence microscopy images were taken using 20x magnification and scale bars represent 100μm. Fluorescence intensity is presented as its median value, and error bars represent the associated 95% CI limits. Typical data is shown.

**A**

|  | Brightfield | Fluorescein | Propidium Iodide |

Media control
P-PQ1-1107
P-1107
PQ1-Aldox-1304
PQ1-Alex-904

**B**

FL fluorescence intensity per cell (AU)

***p≤0.001 compared to media control

Media control, P-PQ1-1107, P-1107, PQ1-Aldox-1304, PQ1-Alex-904

**C**

FL fluorescence intensity per cell (AU)

***p≤0.001 compared to media control

Media control, P-PQ1-1107, P-1107, PQ1-Aldox-1304, PQ1-Alex-904

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**Figure 2: Effect of MPS-treatment on cell viability. (A)** L929 cells stained with fluorescein and propidium iodide after treatment with 0.01% (w/v) BKC for 10min. **(B)** Metabolic activity in L929 cells assessed using an MTT assay after exposure to MPS with or without 0.01% w/v fluorescein or (**C**) metabolic activity assessed using an MTT assay in HC cells. Fluorescence microscopy images were taken using 10x magnification and scale bars represent 100μm. All metabolic activity data is presented as a percentage relative to media control and error bars represent 95% CI limits. Data is representative of several experiments.
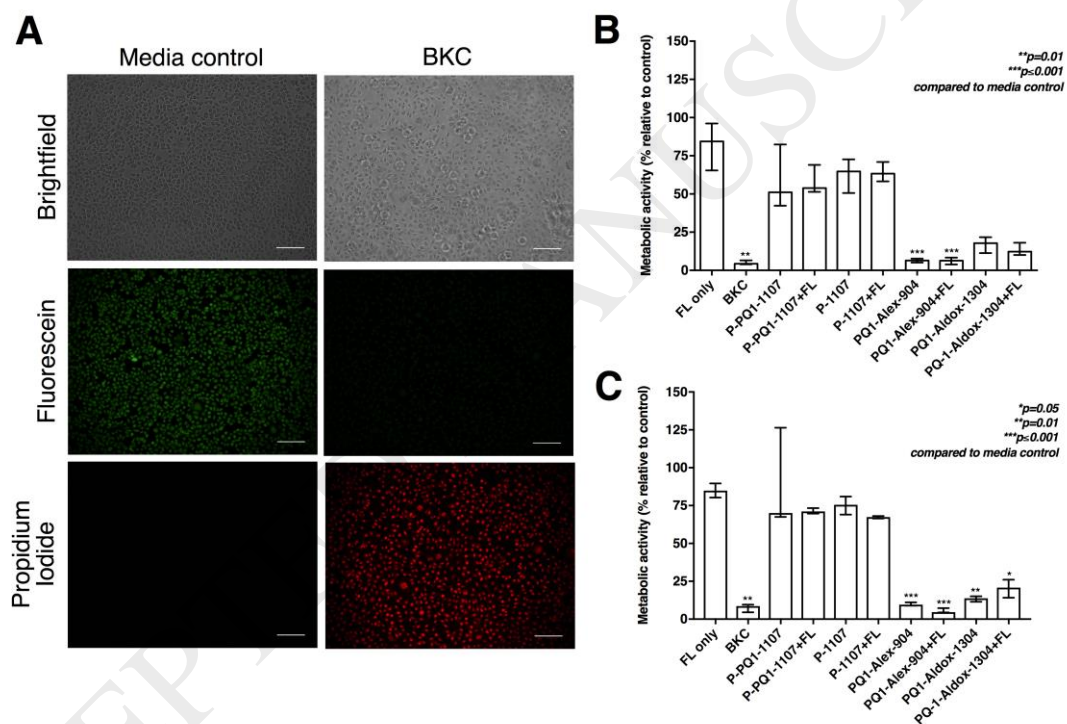
**Figure 3: MPS treatment can cause membrane blebbing.**

**(A)** Confocal microscopy images of L929 and HC cells after MPS treatment and exposure to 0.01% w/v fluorescein. 'Blebbing' was consistently observed after P-PQ1-1107 treatment, though not P-1107 treatment, for both cell types. Confocal images were taken using 40x magnification, and scale bars represent $10\,\mu$ m and $50\,\mu$ m for L929 and HC cells respectively. **(B)** L929 cells treated with MPS and fluorescein and immunostained for cleaved caspase-3. Fluorescent microscopy images were taken using 20x magnification and scale bars represent $100\,\mu$ m. All images are representative of several fields of view.
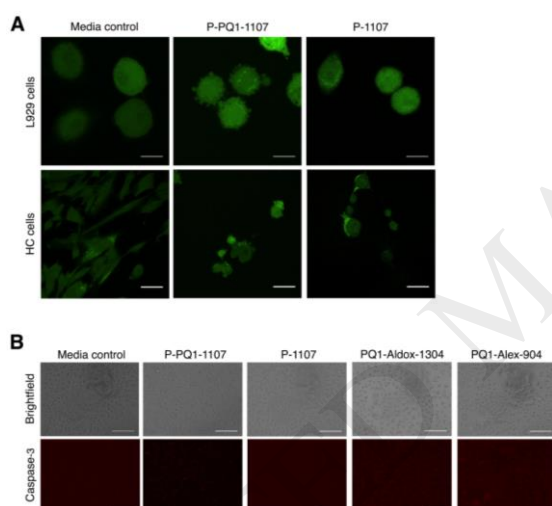
**Figure 4: Dynamin inhibition reduces fluorescein uptake.** **(A)** Effect of dynasore on the intensity of fluorescein uptake by L929 cells after treatment with MPS or **(B)** for HC cells. To confirm fluorescein uptake inhibition was not caused by a non-specific cytotoxic effect of dynamin-inhibition, the metabolic activity of L929 and HC cells respectively was measured after treatment with MPS, dynasore and fluorescein as above. No significant difference was seen between treatments in **(C)** L929 and **(D)** HC cells when compared to control; typical data shown. Metabolic activity is presented as a percentage relative to control and error bars represent the associated 95% CI limits.
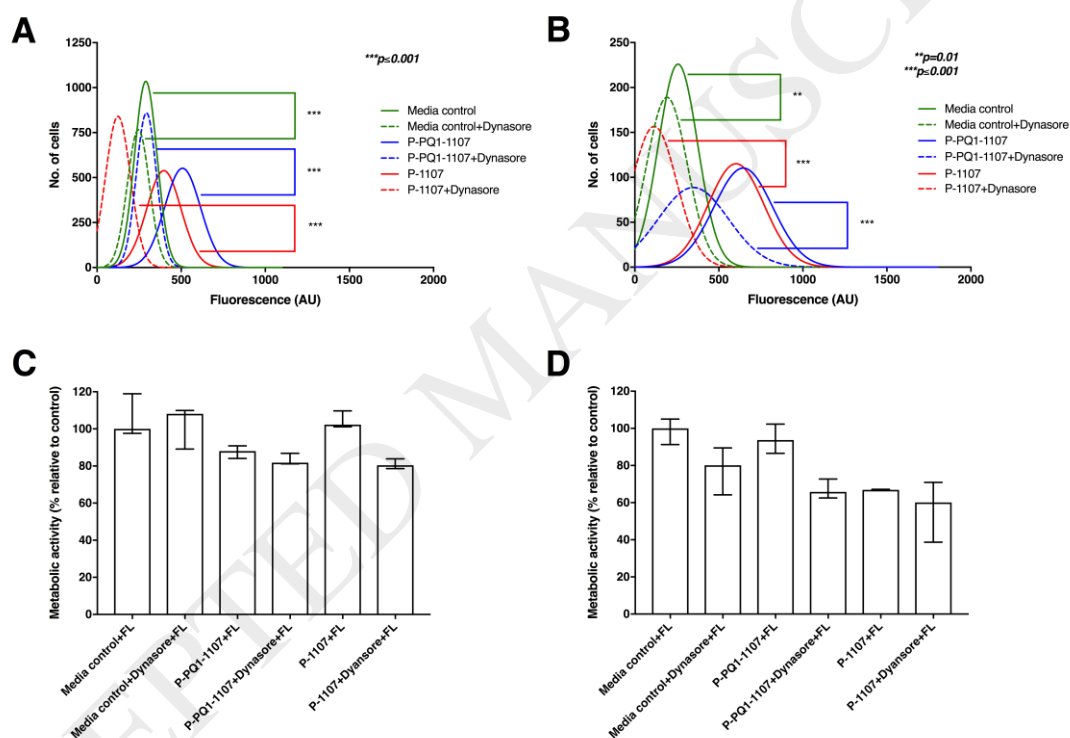
**Figure 5: Role of MPS surfactants in increasing fluorescein uptake. (A)** L929 or **(B)** HC cells were treated with MPS or surfactants and exposed to fluorescein before data acquisition by HCA. Fluorescein fluorescence intensities are presented as median intensity values. **(C)** Effect of the surfactant Triton X-100 on fluorescein uptake by HC cells. We examined whether the increased fluorescein fluorescence after treatment with Tetronic 1107 may reflect increased metabolic activity. **(D)** and **(E)** show the metabolic activity of L929 cells (n=4 wells) and HC cells (n=3 wells) respectively, after MPS or surfactant treatments, or **(F)** after Triton X-100 treatment of HC cells (n=4 wells). Metabolic activities are presented as percentage relative to media control. All error bars represent the associated 95% CI limits. Typical data shown.
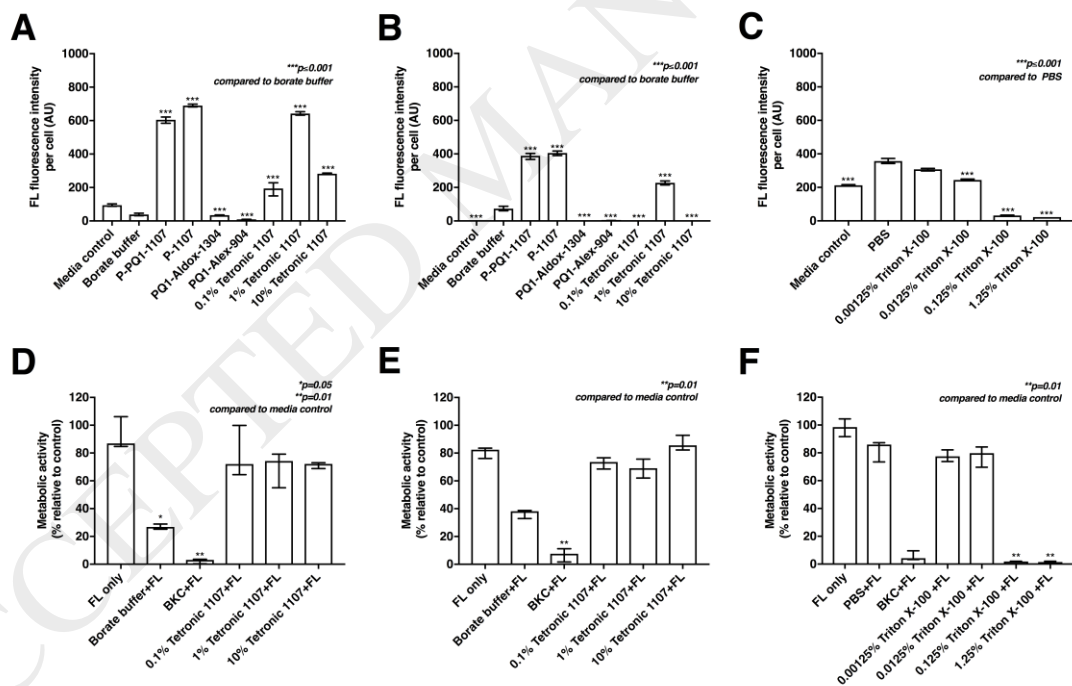
**Table 1: MPS formulations.** MPS are referred to by biocide and surfactant combinations. P, PQ1, Aldox, and Alex refer to the biocides PHMB, Polyquaternium-1, Aldox and Alexidine dihydrochloride respectively. 1107, 1304 and 904 refer to surfactants Tetronic 1107, Tetronic 1304 and Tetronic 904 respectively.

| MPS | Manufacturer | Biocide(s) | Surfactant |
|---|---|---|---|
| **Biotrue** <br> P-PQ1-1107 | Bausch+Lomb | Polyquartenium-1 (0.0001%), PHMB (0.00013%) | Tetronic® 1107 (1.0%) |
| **ReNu Sensitive** <br> P-1107 | Bausch+Lomb | PHMB (0.00005%) | Tetronic® 1107 (1.0%) |
| **Opti-free Replenish** <br> PQ1-Aldox-1304 | Alcon | Polyquaternium-1 (0.001%), ALDOX (0.0005%) | Tetronic® 1304 (0.05%) |
| **Complete Revitalens** <br> PQ1-Alex-904 | AMO | Polyquaternium-1 (0.0003%), Alexidine dihydrochloride (0.00016%) | Tetronic® 904 (0.1%) |

**Table 2: Composition of Tetronic® surfactants in the MPS formulations used in the study.** The number of ethylene oxide (EO) and propylene oxide (PO) units per block is shown (Alvarez-Lorenzo et al. 2010).

| Tetronic | Mw (Da) | EO units per block | PO units per block | Hydrophilic-Lipophilic balance (HLB) |
|---|---|---|---|---|
| 904 | 6700 | 15 | 17 | 12-18 |
| 1107 | 15000 | 60 | 20 | 18-23 |
| 1304 | 10500 | 21.4 | 27.1 | 12-18 |