Hypericin incorporation and localization in fixed HeLa cells for various conditions of fixation and incubation

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Hypericin is a photosensitizer expressing high affinity for cancerous cells *in vivo*. Diagnosis of cancer based on hypericin fluorescence imaging has been successfully assessed in several clinical trials. Our final objective will be to evaluate the potential of hypericin fluorescence imaging to

10 improve the efficacy of cervical cancer diagnosis performed on fixed cell smears obtained from liquid-based cytology. For this purpose, the mechanism of hypericin incorporation and localization in fixed HeLa cells using different incubation media and fixation conditions was investigated. Since the duration of fixation may play an important role, the influence of fixation time on hypericin incorporation in fixed HeLa cells was studied. The uptake and distribution of hypericin

15 in fixed HeLa cells were found strongly dependent on the hypericin incubation medium: for a polar organic solvent such as the alcohol-based fixative, the localization was essentially perinuclear and nuclear; for cell culture medium supplemented with serum, the localization was cytoplasmic and non-specific; the highest incorporation was observed for the culture medium serum free but mainly as non-fluorescent aggregates. The hypericin aggregation in the incubation medium, the passive

20 diffusion and the partitioning between the cells and hypericin carriers seemed to be the major factors accounting for these results. The localization was found weakly dependent on fixation time whereas fluctuations of hypericin fluorescence at short fixation time and stabilization after two days of fixation were observed. These results suggest that the fixed cells reached a steady state after two days of fixation.

25 1   Introduction

A large number of fluorescent probes are of common use in cellular biology for labeling live cells, allowing functional and morphological studies on single live cells by fluorescence microscopy [1, 2]. Among these dyes, a limited number are used

30 in human beings for in vivo diagnostic applications [3-5]. One particular application employs photosensitizers, taking advantage of their preferential localization in tumour sites after administration to the patient. Therefore, cancerous cells and tissues can be distinguished from normal ones by measuring

35 the photosensitizer fluorescence emission which will be higher in cancerous sites. This has led to a new method of cancer detection named photodynamic diagnosis (PDD) [6].

Many studies were conducted in order to investigate the incorporation mechanisms of dyes in living tissues and live

40 cells [7-11]. Fixed cells are very common specimens for immunological labeling and morphological staining for cytological diagnosis [12, 13]. In the latter category, the best-known cytological test is the Papanicolaou (PAP) staining which is used for cervical cancer diagnosis. This test consists

45 of collecting exfoliated cells from the cervix, preserving them in a fixative then staining and examining them visually under microscope. The detection and grading of cancers are based on several cellular morphological anomalies [14]. In the second half of the twentieth century, screening programs based on this test

50 made early diagnosis possible, resulting in a considerable reduction in invasive cervical cancer incidence and mortality

in developed countries. Despite the effectiveness of the method, the interpretation can be affected by morphological anomalies which do not give a clear confirmation for diseases

55 (ASCUS: Atypical Squamous Cells of Undetermined Significance) [14]. Research is still ongoing in order to improve the test efficacy in terms of sensitivity and specificity.
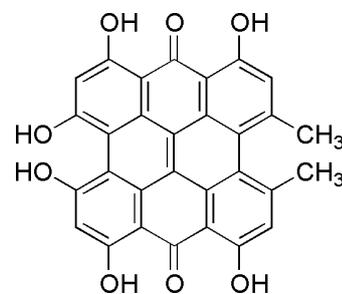

Figure 1: Hypericin

60 The liquid-based cytology was introduced as a way to improve the test performance. Rather than being spread and fixed onto a glass slide immediately after scraping, the exfoliated cells are transferred to a fixative solution and transported to the laboratory for slide preparation and analysis. The liquid-based

65 method allows mono-layer smears and automation of the preparation. It reduces about 80% of inadequate sampling [14]. Moreover, bio-molecular analysis can be performed.

Hypericin (Hyp), a polycyclic quinone (Fig. 1), is a naturally occuring photosensitizing pigment extracted from St. John's

wort [15, 16]. Hypericin fluoresces brightly in the 600-650 nm spectral region and is more photostable than most other photosensitizers. Its selective accumulation in live malignant tissues made it a good candidate for oncological applications of hypericin-based PDD [17, 18]. The reasons accounting for the preferential hypericin accumulation in tumors are likely to be related to its transporters such as Human Serum Albumin and lipoproteins [11]. According to several clinical studies, the sensitivity of bladder cancer diagnosis with hypericin ranges from 82% to 94% and the specificity from 91% to 98.5% [19-21]. Other studies extended the use of hypericin-based PDD to other cancer types such as oral cavity squamous cell carcinomas [22] and pancreatic tumours [23]. All these studies were carried out *in vivo*; some *ex vivo* studies were reported but exclusively on live cells [8, 24].

Recently, it was patented that this photosensitizer incorporates very rapidly in tumorous cells which are placed in suspension in a fixing solution [25]. This patent claims that hypericin, when added to HeLa cells (a line of human epithelial cervical cancer) fixed in a cytological fixing agent, already including normal cells from a cervical smear, exhibits a stronger fluorescence emission in the tumorous cells than in the normal ones. We observed the same result of a stronger hypericin fluorescence in fixed HeLa cells compared to normal fixed cells in the same mixture (unpublished results). So, hypericin appears as a potent photosensitizer for in vitro diagnosis of malignant tumors such as cervical cancer. If the incorporation mechanisms of fluorescent compounds in living cells were studied by many authors, it is not the case for fixed cells. Obviously, the incorporation processes involved cannot be explained by the same mechanisms in live and fixed cells.

Considering, the high sensitivity of fluorescence-based diagnosis method and the current sensitivity of PAP cytological test, around 70 % [26], it would be of interest to assess the use of hypericin fluorescence in a cytological test. Our final aim is to evaluate the potential of hypericin to improve the efficacy of cervical cancer diagnosis performed on fixed cells obtained from liquid-based cytology preparation.

For this purpose, first of all, it is essential to understand the mechanisms of hypericin incorporation in fixed cells, which is the objective of the study reported here. Indeed, the mechanisms underlying the hypericin accumulation in live cells can actually not be applied to fixed cells.

Hypericin incorporation and localization in fixed HeLa cells – a model of cervical cancer cells – were investigated using various incubation and fixation conditions. In clinical practice, the time interval between the beginning of cells fixation and the cytological analysis can vary between a few hours and one week. Therefore, the influence of the fixation time on the hypericin incorporation in fixed cells was also investigated. For these studies, we used fixatives (Novacyt, Vélizy-Villacoublay, France) which are clinically employed for liquid-based cytology.

2 Materials and methods

2.1 Cell culture

HeLa cells (human cervix adenocarcinoma from ATCC, containing human papilloma virus 18) were cultivated routinely as monolayer in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine (862 mg/l), sodium pyruvate (110 mg/l), glucose (4500 mg/l), supplemented with penicillin (50 units/ml), streptomycin (50 µg/ml) and 10% foetal bovine serum (FBS). For the experiments, cells were seeded at a density of 20,000 (cells/cm^2) on a glass slide in 35 mm Petri dishes about 48 h before experiments.

2.2 Fixatives

Alcohol-based fixative containing ethanol and isopropanol and the fixative of the same composition supplemented with formaldehyde (<1%) were supplied by Novacyt (Vélizy-Villacoublay, France). From now "the fixative" refers to the one without formaldehyde unless otherwise specified.

2.3 Hypericin

Hypericin was purchased from Molecular Probes, Oregon, USA. A stock solution was prepared in dimethyl sulphoxide (DMSO) at a concentration of 2 mM. For the experiments, hypericin was further diluted in the incubation medium (the fixative, DMEM + 10% FBS or DMEM without FBS) to a concentration of 2 µM. From now on, hypericin dissolved in the fixative will be denoted as FIX, in DMEM + 10% FBS as DMEM-S and in DMEM serum free as DMEM-SF. Experiments were carried out in subdued light conditions.

2.4 Cell fixation and fixed cell incubation with hypericin

The culture medium was discarded and 2 ml of fixative were added to the Petri dishes. After certain fixation time (5 min, 15 min, 30 min, 1 h, 3 h, 6 h, 24 h, 2 days (d), 5 d and 7 d), the fixative was removed. The cells were incubated during 30 min with 2 ml of hypericin 2 µM dissolved in the three media described previously. The cells were washed twice with 1 ml of Hank's balanced salt solution (HBSS) before hypericin extraction or being mounted for fluorescence imaging. All the incubations were carried out at room temperature. The cells were fixed in the fixative without formaldehyde unless otherwise stated.

2.5 Cellular extraction of hypericin

The cells were scrapped in 900 µl of tri-distilled water leading to total cell lysis. 100 µl of a 3% Triton X-100 aqueous solution were added and the disrupted cell solution was collected. This solution was used for fluorescence measurement of hypericin (excitation wavelength: 595 nm, spectral detection range: 630-660 nm) on an Aminco Bowman Series 2 Luminescence Spectrofluorometer (Bioritech, Chamarande, France). The signal at the 650 nm peak was used as the fluorescence intensity value. Then, 150 µl were used for protein determination according to the method of Lowry [27]. Solutions of known hypericin concentration in Triton X-100 0.3% were used as standard for hypericin quantification. Human serum albumin was used as standard for protein determination. Data is expressed as µmole of hypericin per gram of protein. All the experiments were done in triplicate.
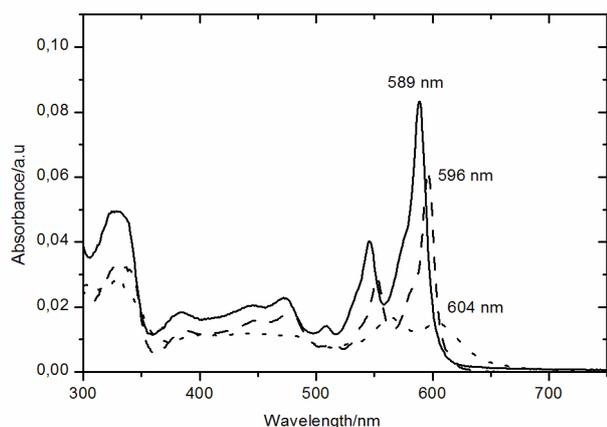
2.6 Hypericin fluorescence imaging

Figure 2: Absorption spectrum of hypericin in different media: the fixative (full line), DMEM + 10% FBS (dashed line) and DMEM serum free (dotted line). Hypericin concentration: 2 μM.

The setup comprises a large field Nikon Eclipse TE300 inverted microscope equipped with a high numerical aperture phase oil objective (100X Plan Fluor Ph3 DLL Oil NA=1.3 Nikon, France), a X-Cite 120PC Metal Halide lamp as excitation source and a Photometrics CoolSnap HQ 12 bits Camera for image acquisition. A set of neutral density filters was used to attenuate excitation (ND16, ND8 and ND4). For fluorescence imaging of hypericin, the filter set was chosen after preliminary spectral studies of hypericin in the three media (Fig. 2). The system employed a 560/40 nm excitation filter, a dichroïc mirror with a cut-on wavelength of 595 nm and a 630/60 nm emission filter. Before each experiment, the excitation intensity was measured by a Chroma fluorescence reference slide. Fluorescence images of hypericin were acquired with an integration time of 1 second using Metamorph software supplied by Universal Imaging Corporation (Roper Scientific, France). The cells were immersed in HBSS during observation.

2.7 Image analysis

ImageJ (Wayne Rasband, National Institutes of Health, USA) was used for image analysis. The images were corrected for dark noise and inhomogeneous illumination. For each slide of cells, images of about 70 cells were acquired. The cellular contours were delineated manually and the fluorescence intensity of each cell (sum intensity of all pixels over the number of pixels) was calculated. Then the average of fluorescence intensity of these 70 cells was calculated and normalized by the excitation intensity. The data presented is the mean (±S.D.) of 3 separate samples.

2.8 Absorption spectrum of hypericin

Absorption spectra were recorded using an Uvikon 932 Double Beam UV/VIS Spectrophotometer (Bio-tek Kontron, Milano, Italy) at room temperature.

3 Results

3.1 Hypericin incorporation in fixed HeLa cells as a function of fixation time, comparison between different incubation media
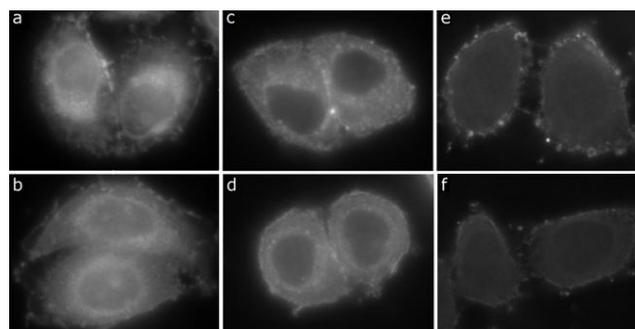

Figure 3: Subcellular localization of hypericin in fixed HeLa cells with hypericin dissolved in different incubation media. a and b: for FIX with fixation time of 5 min and 24 h respectively. c and d: for DMEM-S with fixation time of 5 min and 24 h respectively. e and f: for DMEM-SF with fixation time of 5 min and 24 h respectively. The visualization parameters were set to better show hypericin distribution but do not reflect the relative difference of fluorescence intensity between the three incubation media.

The incubation medium is known to affect the hypericin uptake and distribution in live cells [21,22,23] and is expected to have an influence in fixed cells as well. Therefore we studied hypericin incorporation and localization using three different incubation media: the fixative, DMEM and DMEM supplemented with 10% FBS. The two last media were used for studying hypericin incorporation and localization in live cells [28-30]. The properties of hypericin in the fixative are expected to resemble those in a polar organic solvent since the fixative contains an important proportion of ethanol and isopropanol.

3.1.1 Fluorescence imaging of hypericin.

Hypericin localization. After 30 min of incubation with hypericin, a bright fluorescence in fixed HeLa cells was observed in the case of FIX and DMEM-S while conversely in the case of DMEM-SF the fluorescence was weak. The fluorescence pattern changed slightly with respect to fixation time for all three media (Fig. 3). For the three incubation media, the fluorescence localization was different.

In the case of FIX (Fig. 3, a and b), the perinuclear fluorescence was markedly brighter than other parts of the cells; the cytoplasm had some discrete particle-like staining; the perinuclear staining decreased slightly with fixation time; some regions of the nucleus were also stained; these regions were seen as dense regions in phase-contrast microscopy (images not shown). For DMEM-S (Fig. 3, c and d), the perinuclear staining was not notable; all the cytoplasm area seemed to be stained equally; the nuclear fluorescence was faint. In the case of DMEM-SF (Fig. 3, e and f), the fluorescence intensity decreased from the plasma membrane to the nucleus. Whatever the incubation medium used, small vesicle-like structures markedly stained were found near the plasma membrane and sometimes in the cytoplasm; the discontinuity of the plasma membrane staining could be noted.

Quantitative imaging. Figure 4 presents the results obtained by fluorescence imaging of hypericin. For all the media studied, fluctuations of fluorescence intensity at short fixation time (< 3 h) were observed, followed by a weak evolution after 3 h of fixation. The mean fluorescence intensity in fixed HeLa cells
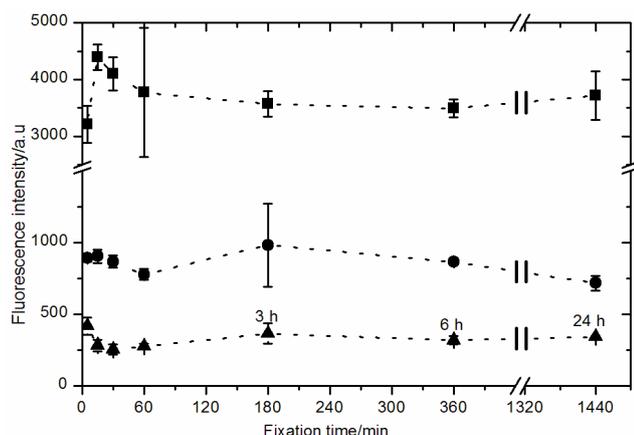
Figure 4: Hypericin fluorescence intensity in fixed HeLa cells versus fixation time measured by fluorescence imaging in the three cases: FIX (square), DMEM-S (circle) and DMEM-SF (triangle). The figure uses a horizontal break from 420 min (7 h) to 1320 min (22 h) and a vertical break from 1500 to 2500.
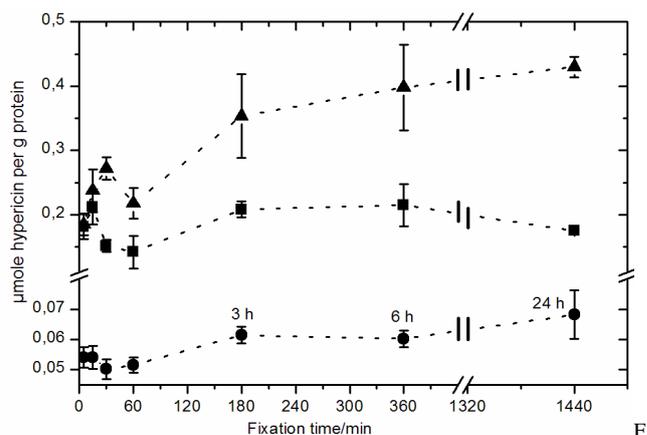
Figure 5: Hypericin fluorescence intensity in fixed HeLa cells versus fixation time measured by extraction in the three cases: FIX (square), DMEM-S (circle) and DMEM-SF (triangle). The figure uses a horizontal break from 420 min (7 h) to 1320 min (22 h) and a vertical break from 0.08 to 0.11.
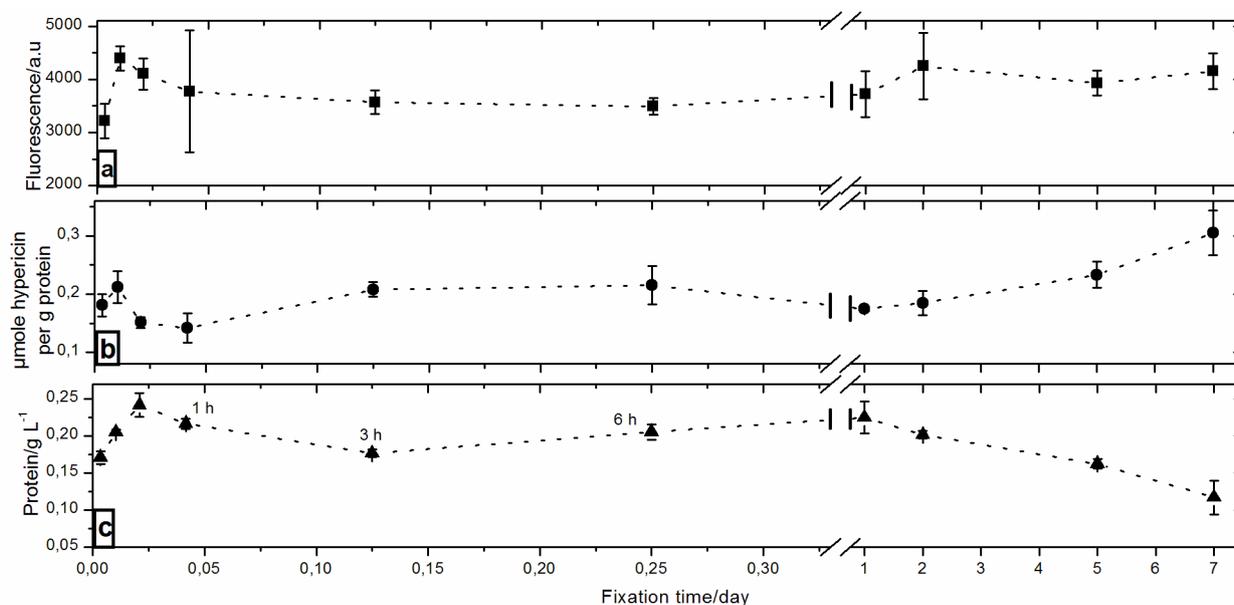

Figure 6: One week study of hypericin fluorescence intensity obtained by fluorescence imaging (a), by extraction (b) and evolution of protein concentration (c) in fixed HeLa cells as a function of fixation time. The fixative was used as incubation medium. Horizontal break used from 0.33 d (8 h) to 0.75 d (18 h).

for FIX was about four times greater than DMEM-S and ten times greater than DMEM-SF.

3.1.2 Hypericin extraction.

In hypericin extraction (Fig. 5), the fluctuations for a fixation time shorter than 3 h were also observed and followed by a weak evolution after 3 h of fixation. A decrease of hypericin incorporation at about 1 h followed by an increase at 3 h of fixation could be noted. This trend could also be seen by fluorescence imaging but less pronounced. The largest hypericin incorporation was observed in the case of DMEM-SF. The hypericin incorporation in the case of FIX was about three times greater than DMEM-S.

3.2 Study up to one week of hypericin incorporation in fixed HeLa cells as a function of fixation time

As mentioned above, in clinical routine, the cells may stay in the fixative several days and even weeks before being analyzed. Therefore, our study of hypericin incorporation versus fixation duration was extended to one week. The fixative was the most effective medium for observing hypericin fluorescence in fixed cells. It is also convenient for clinical routine since the cells can be incubated directly by adding hypericin to the preservation vial. Thus, we used the fixative as medium for hypericin incubation.

Figure 6a shows the fluorescence intensity obtained by fluorescence imaging. The result up to 24 h was presented in the previous section (Fig. 4). The fluorescence intensity increases from 1 to 2 days then is quasi-stable from 2 to 7 days of fixation. The result obtained by extraction (Fig. 6b) was different from fluorescence imaging: hypericin incorporation (normalized by cellular protein content) increases with fixation duration from one day to one week
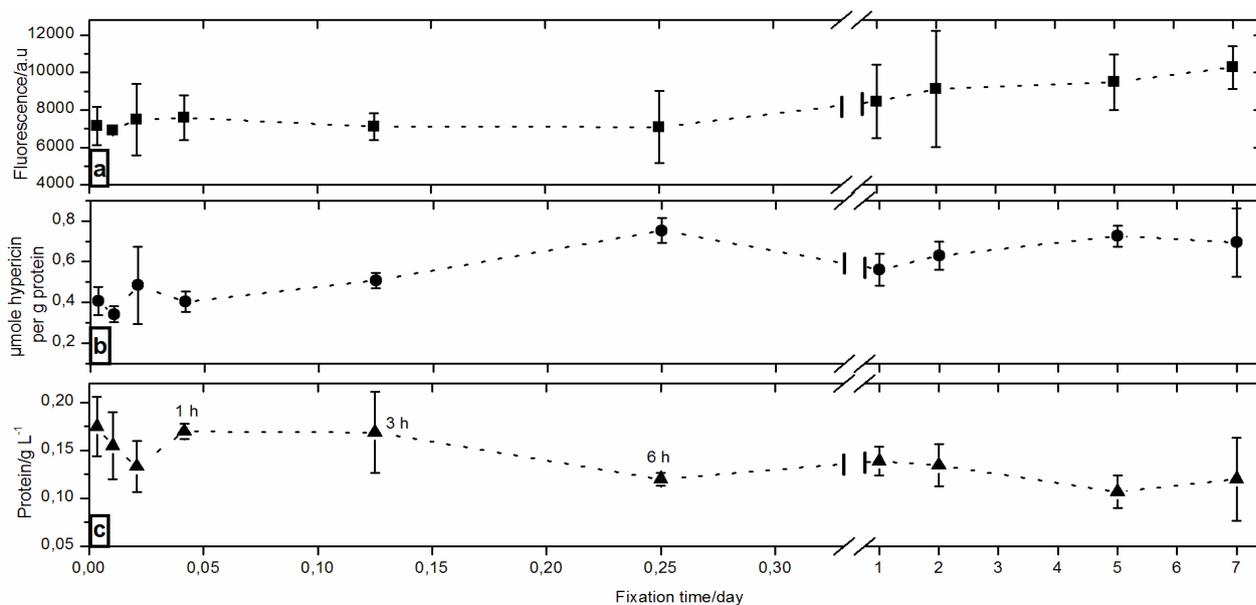
Figure 7: One week study of hypericin fluorescence intensity by fluorescence imaging (a), by extraction (b) and evolution of protein concentration (c) in fixed HeLa cells as a function of fixation time. The cells were fixed in alcohol-based fixative with added formaldehyde. The fixative without formaldehyde was used as incubation media. Horizontal break used from 0.33 d (8 h) to 0.75 d (18 h).

without stabilization beyond 2 days. This increase seems to correlate with the decline in the protein concentration after one day of fixation (Fig. 6c) whereas at fixation time shorter than one day, no correlation was observed.

3.3 Hypericin incorporation in HeLa cells fixed with the fixative with formaldehyde

Formaldehyde is known to create cross-links between proteins [31, 32]. The intra- and intermolecular cross-linking stabilizes the cellular proteins. To verify if the lost of proteins observed earlier (Fig. 6c) was related to the increase of hypericin incorporation in hypericin extraction (Fig. 6b), the alcohol-based fixative was replaced by the same fixative supplemented with formaldehyde (see 'Materials and Methods') and the experiment described in the section 3.2 was repeated. The fixative with formaldehyde was only used for cell fixation. Hypericin was dissolved in the fixative without formaldehyde for hypericin incubation. All other experimental parameters were kept identical to those used in section 3.2.

In fluorescence imaging (Fig. 7a), a similar behaviour of hypericin incorporation in fixed HeLa cells to those in section 3.2 (Fig. 6a) was observed. In hypericin extraction (Fig. 7b), the evolution of hypericin incorporation after one day of fixation was similar to fluorescence imaging (Fig. 7a). The hypericin incorporation was higher than measured in section 3.2 (Fig. 6a, 6b, 7a and 7b). The decrease of protein concentration with fixation time longer than one day was no longer notable (Fig. 7c).

4 Discussion

The properties of hypericin in water and in organic solvents have been reported by several authors: hypericin is soluble in monomer form in polar organic solvents and shows well resolved absorption and fluorescence spectra up to concentration of 10^{-3} M [33]. Hypericin is sparingly soluble in water and forms non-fluorescent dimers at low concentration (5×10^{-7} M to 5×10^{-5} M) [34] and oligomers/aggregates at higher concentration [33]. The fluorescence of hypericin in solution is exclusively due to the monomer form. The absorption spectrum is highly affected by the aggregate formation [33-35]. In our experimental conditions, as expected, we found that the spectral properties of hypericin in the fixative and in DMEM + 10% FBS were similar to a polar organic solvent and in DMEM similar to water (Fig. 2).

Several studies demonstrated that a major part of hypericin binds to low density lipoproteins (LDL), in a lesser extent to high density lipoproteins and very low density lipoproteins in human plasma [29, 36]. Hypericin can also make a 1:1 complex with serum albumin [37] Hypericin (Hyp) binds with LDL in monomer form at low Hyp/LDL ratio but forms aggregates and self-quenches in LDL at high Hyp/LDL ratio [38, 39]. At high concentration of hypericin in LDL, the fluorescence may decrease even when the monomer form still dominates because of fluorescence quenching by the aggregates at the proximity of the monomers [38,39]. In our conditions, up to 6 µM of hypericin in DMEM + 10 % FBS, the hypericin monomers still seemed to predominate but the fluorescence started decreasing after 3 µM probably due to the fluorescence quenching (data not shown). At the hypericin concentration of 2 µM that we used, hypericin should exist essentially as monomer in the presence of serum proteins and the fluorescence quenching by hypericin aggregates should be negligible.

Hypericin is known to bind to lipid membranes in live cells. However the distribution of hypericin among organelles depends on the cell line used and on the incubation time [40]. In HeLa cells, hypericin has been found to localize in the endoplasmic reticulum (ER) and the lysosomes after

incubation, in the ER and Golgi apparatus after 24 h of incubation [41] and not in the mitochondria [36]. Weber *et al.* showed that the intracellular membranes staining was probably 16 h of due to the active transport across the cell membrane since an inhibitor of endocytosis or membrane cycling between the ER and Golgi counteracted the intracellular accumulation of hypericin in cultured cells [42].

For FIX, a perinuclear staining in fixed HeLa cells was observed. This pattern seems to result from the free access of hypericin to the perinuclear regions after plasma membrane disruption. The fluorescence at some dense areas of the nucleus was also noted. The nuclear staining of fixed HeLa cells should be explained by hypericin existence as monomers in the fixative which allows penetration and free diffusion in the nucleus. It can be noted that no nuclear staining was observed in live cells [36, 40, 41].

For DMEM-S, hypericin seemed to be uniformly distributed in the cytoplasm area of fixed HeLa cells. The nucleus was not stained. It is known that serum proteins act as carriers of hypericin. This hypericin localization probably results from the diffusion pattern of serum proteins in the fixed cells. Since the serum proteins are much bigger than hypericin monomers, their limited diffusion should explain the lower hypericin incorporation compared to the case of FIX (Fig. 4 and Fig. 5) and the absence of nuclear fluorescence. The monomer form of hypericin is dominant in fixed HeLa cells in this case but a minor fraction of aggregates cannot be excluded.

For DMEM-SF, the fluorescence imaging results show very weak fluorescence (Fig. 4) which implies a low incorporation of hypericin in fixed HeLa cells. Surprisingly, the hypericin quantity measured by extraction (Fig. 5) is the highest among three media used which implies the highest level of hypericin incorporation. This apparent contradiction suggests that the majority of hypericin exists as non-fluorescent aggregates in fixed HeLa cells. There should be a small proportion of hypericin under monomer form which accounts for the observed fluorescence because the aggregates of hypericin are totally non-fluorescent.

Several groups have compared the incorporation of hypericin in live cells using hypericin dissolved in cell culture medium with and without serum. Hypericin uptake was found higher when hypericin was dissolved in a medium serum free [28, 29]. These observations are in good agreement with our results by hypericin extraction (Fig. 5) and our hypothesis of hypericin existence as aggregates in the case of DMEM-SF. In fact, fluorescence imaging measures only hypericin monomers since they are unique fluorescent species whereas hypericin extraction quantifies all hypericin content as the non-fluorescent aggregates are converted to monomers.

We suggest that two factors might explain the higher incorporation of hypericin in the case of DMEM-SF compared to DMEM-S. First, since passive diffusion is the only process which determines hypericin incorporation in fixed cells, this greater incorporation can be partly due to the facilitated diffusion of hypericin aggregates compared to hypericin bound to serum proteins. This hypothesis is supported by the reported values of serum proteins molecular weight (*e.g.* human LDL between 1.8 and 2.8 million [43]) which are much

greater than the hypericin dimers and aggregates (hypericin molecular weight: 505 and apparent molecular mass of hypericin aggregates of more than 2000 [33]). The other important factor may be the partitioning of hypericin between the cells and hypericin carriers. For DMEM-SF, hypericin partitions between the cells and DMEM. In the case of DMEM-S, the cells must compete with the serum proteins. The binding of hypericin to serum proteins (in human plasma) suggests that the later constitutes a more hydrophobic environment compared to DMEM. Thus, we believe that the hypericin partitioning to the cells is more important in the case of DMEM-SF than DMEM-S.

In live cells, it is known that the aggregation of hypericin does not impair its incorporation and the aggregates are monomerized after incorporation [28, 30]. On the contrary, in fixed HeLa cells, only a small proportion of aggregates incorporated were monomerized. However, the comparison between live and fixed cells is difficult since the fixation process strongly alters the cellular membrane system.

Regarding the influence of fixation time on hypericin incorporation in fixed HeLa cells, a stabilization of hypericin incorporation after two days of fixation was observed by fluorescence imaging. However, hypericin extraction showed inconsistent result: hypericin incorporation increases after two days up to seven days of fixation. A significant lost of proteins of the sample was noted at the same time. This lost can probably account for the observed increase of hypericin incorporation in extraction results since hypericin fluorescence was normalized by protein content. To confirm this hypothesis, we used the fixative supplemented with formaldehyde – a protein cross-linking agent – to fix HeLa cells and the experiment was repeated. The protein lost observed previously was not seen anymore. The intra- and intermolecular cross-linking seemed to prevent the lost of proteins into the fixative and due to washing steps. Fluorescence imaging and extraction of hypericin showed similar results (Fig. 7a and 7b) which confirm the stabilization of hypericin incorporation after two days of fixation. The fluorescence intensity was somehow higher in the case of fixative with formaldehyde. These results suggest that the properties of fixed cells do not evolve significantly after two days of fixation.

5 Conclusion

The mechanism of hypericin incorporation and localization in fixed cells are found strongly dependent on hypericin state in the incubation medium, *i.e.* the binding of hypericin to carriers and its aggregation. For all the fixation times investigated, the hypericin localization in fixed cells was found quite different from live ones. The difference of hypericin incorporation and localization compared to live cells is not only due to the simple halt of living process but also due to the alterations of cellular components by the fixation. The fact that hypericin incorporation is stabilized after two days of fixation suggests that the fixed cells reach a steady state beyond this fixation time.

This study is the first step of the evaluation of the hypericin fluorescence potential for improving the efficacy of cervical

cancer diagnosis performed on fixed cells. Moreover, since most of cytological diagnoses are carried out on fixed cells in practice, our results give a better insight into staining process of fixed cells.

Acknowledgments

Notes and references

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