Fluid-mediated intracellular delivery: from a Single Channel to a Porous Cartridge

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***Abstract*—Red Blood Cells (RBCs) are promising drug delivery systems for different types of molecules whose internal delivery can be obtained by exploiting several approaches. The possibility of using microfluidic shear stress for this aim, preserving the integrity of the cellular membrane, has already been proven in a single-channel configuration. However, a single microfluidic channel is inadequate for clinical use due to the small output, so newly conceived scaled-up devices are needed. This work wants to test a prototype of a microfluidic cartridge for intracellular delivery of drugs in erythrocytes.**

The cartridge prototype is 3D-printed and made of parallel interconnected channels. The new device is here tested with bovine RBCs to verify the encapsulation performance obtainable through its use. The experimental setup comprises a syringe pump connected to the chip/cartridge, which, in its time, is connected to a reservoir. The proof of concept of the intracellular delivery in RBCs has been carried out by using fluorescent probe molecules (Fitch-Dextran 40kDa), comparing the performances obtained with the cartridge with the ones with the single channel, both in terms of quantity of probe molecules encapsulated by the RBC during the flow throughout the cartridge.

The intracellular delivery rate obtained using the porous cartridge is statistically equivalent to the one with the single channel. The experimental tests confirm then the new cartridge's potential for treating blood samples, preserving cell suspension, and favoring intracellular delivery in bovine cell RBCs.

***Keywords*— Intracellular delivery, erythrocytes, microfluidics, encapsulation.**

I. Introduction

Modifying the pharmacokinetics and biodistribution of therapeutic agents represents a strategic approach to enhancing therapeutic efficacy and minimizing adverse effects. Existing artificial drug delivery systems are based on various technologies, ranging from macromolecules such as micelles and liposomes, which are characterized by low systemic toxicity, to nanoparticles and peptides, which can enhance drug specificity through selective binding [1, 2]. Some of these delivery systems have already received regulatory approval, while others are currently undergoing clinical trials [3], [4]. Nevertheless, several challenges remain. First, material-related toxicity continues to pose a significant concern for many nanomaterials, particularly concerning inflammatory responses, hemocompatibility, and potential effects on reproductive systems [5]. Furthermore, the mechanisms of carrier absorption, distribution, and metabolism remain insufficiently understood [6], necessitating considerable efforts to develop reliable and effective products [7]. The use of autologous cells opens up many possibilities to revolutionize the field of intracellular delivery of various types of materials. The approach is of high interest for gene editing, cell therapy, and basic research due to the many combinations of both cell types and cargo molecules that can be addressed [7], [8], [9], [10], [11], [12], [13], [14]. Among them, Red Blood Cells (RBCs) are biocompatible and easily available drug carriers [7], [8], [9], [10], [11], [12], [13], [14]. Different techniques have been proposed for the internal delivery of compounds into RBCs. Among them, it has already been proved that a single microfluidic linear straight channel embedded in a PMMA chip allows the internal delivery of drugs into human RBCs with relevant performances [13], [14] by exploiting controlled fluidic shear stresses, even in the presence of proteins [15].

One limit of this approach, as described in the literature [15], is the limited flow rates of the process and the high dilution of the suspension, which is necessary to avoid the systematic sedimentation of cells and the clotting of the channel observed when operating at hematocrit (Ht) higher than 5%. These observations highlight the need for a new device with improved internal fluid dynamics.

A device in the form of a Porous Cartridge (PC) [16], increasing the area of the flow section, can overcome this limit, allowing higher flow rates to be processed. PC can be 3D-printed with additive manufacturing technology [17] and comprises many parallel channels, each one with features and connections favoring intracellular delivery and remixing respectively.

Here, as a proof of concept, we preliminary evaluate its encapsulation performance by using bovine blood, in comparison with the single channel one.

II. Materials and Methods

A. Blood supply

Bovine RBCs are separated from blood slaughterhouse samples from healthy cows (competent veterinary service declaration - Ospitaletto Lodigiano – LO - Italy).

Bovine blood is chosen because it is available and mechanically similar to human blood, as confirmed by its use in mechanical Hemolysis Assays of Blood-Contacting Devices [18].

Blood was treated with Lympholyte® separation media (Cedarlane Labs, Burlington, Canada) to separate plasma and nucleated cells by RBCs. The sample was centrifugated at 3000 rpm for 30 min without brake. The supernatant was removed (separation media, nucleated cells, and plasma). The RBC sample was diluted in Phosphate Buffer Saline (PBS), and the centrifuge was repeated. The supernatant was still

removed.

B. Fabrication of the PCs

The prototypes are fabricated using 3D printing additive manufacturing techniques with PLA (Polylactic acid) [1] [19]. Their internal structure is porous, with open pores features ranging between 50 and 100 µm featuring a structure equivalent to 45 parallel microchannels as the PMMA channel taken as reference.

C. Probe molecule intracellular delivery protocol and experimental setups

Intracellular delivery tests followed a standard and validated protocol [14], [15]. After removing the supernatant, RBCs were suspended at Ht=10% in GASP (PBS + 1 mg/mL of Bovine Serum Albumin - Sigma-Aldrich S.r.l., Milan, Italy), used to model the presence of plasma protein [15]. According to the literature, 40 kDa FITC-dextran (FD40S, Sigma-Aldrich S.r.l., Milan, Italy) was used as a probe molecule to test the encapsulation efficacy and added at 0.4 M concentration (16 mg/mL) [13], [14], [15].

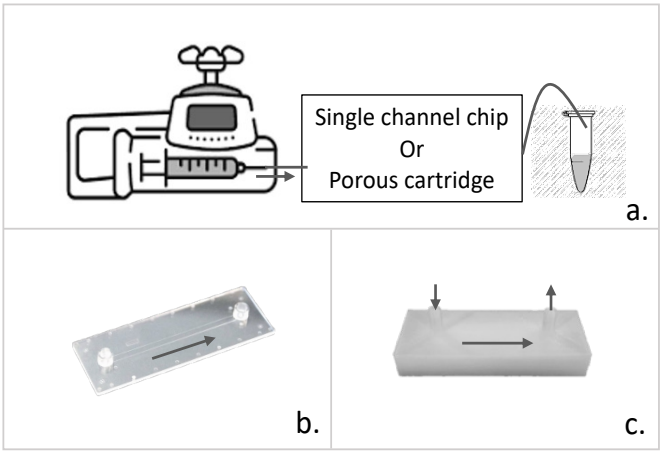

Fig. 1. a. Schematic of the fluidic setup used for internal delivery of FD40 into RBCs. The same syringe pump can be connected alternatively to a single channel chip or to a porous cartridge. The outlet of the component aimed at the internal delivery of the compound is, in both versions, connected to a reservoir. b. Microfluidic single-channel chip. c. Porous cartridge.

Data were obtained from a fluidic setup, as in Figure 1a, in which the single channel chip or the porous cartridge can be alternatively used. In the first case, from the literature [14], [15], the component aimed at the internal delivery of the compounds is a PMMA chip (Microfluidic ChipShop GmbH, Jena, Germany; Figure 1b) with a length of 58,5 mm. A syringe pump (Harvard Apparatus PHD 2000, Massachusetts, USA) pushed the RBCs suspension through the single channel, on the chip, through a customized mini-Luer port (Microfluidic ChipShop GmbH, Jena, Germany). The use of the syringe pump allowed the implementation of a fixed and stable flow rate that was stepwise increased (5-30-50 µL/min). In the second case, the PC (Figure 1c), described in the previous paragraph, is connected to the same pump.

The flow rates studied ranged from 600 to 1300 µL/min, selected to provide a similar cell stress to the single-channel case, taking into account the ratio of the flow section area to the successfully set flow rate in the single channel; the repeated shear stresses related to the repeated inlets and outlets of the porous pathway; and the edge effects of the 3D-printing implying incomplete patency of the layers close to the solid wall. The upper limit is given by the weak sealing of inlet and outlet connections.

D. Intracellular delivery efficiency evaluation

The sample (150 µl) at each flow rate was collected at the outlet of the channel/cartridge, and the dextran still in suspension (not encapsulated in the cells) was removed after centrifugation (3000 rpm for 5 minutes). The RBCs were re-diluted 1:1 in PBS. The sequence centrifugation - supernatant removal was repeated two times. At the end of the washing process, the RBCs of each sample were lysed by using a controlled volume of distilled water to expose in solution the cell's content. The sample was centrifuged, and the supernatant was analyzed by measuring the fluorescence of FITC-dextran released by the loaded erythrocytes after their lysis. These measures were taken using a multifunctional microplate reader TECAN Infinite 200 PRO (Tecan AG, Switzerland). The intensity of fluorescence, the Relative Fluorescent Units (RFU) detected by the photomultiplier, is directly proportional to the concentration (c) of the fluorescent molecule under analysis:

$$RFU = K \cdot c \qquad (1)$$

where K is a constant parameter determined by calibrating the instrument on the particular fluorescent molecule. To create a calibration curve, different samples at a known concentration of dextran are placed in a 96-flat multi-well plate and measured by using a TECAN ® reader. Dilutions in series 1:100 were performed to obtain samples in a range going from $1 \cdot 10^{-1}$ mM to $1 \cdot 10^{-11}$ mM. For each dilution, three wells were filled, and fluorescence values were acquired to build the calibration curve and define K.

The measures taken on loaded RBC samples, as an increase in fluorescence, proportional to the Dextran released after the lysis, must be compared to the negative control (N) and Untreated (UT) samples.

The untreated Sample (UT) was collected from plain distilled water without the addition of lysed RBCs or FD40S. The negative control (N) was, instead, collected by measuring the fluorescence of FD40S released by RBC exposed to the molecule without any flow shear stresses to quantify the contribution of the free diffusion of molecules through the RBC membrane.

Fluorescence intensity values detected by TECAN ® reader all over the analyzed wells were elaborated through the calibration curve, to estimate the related concentration value and the number of molecules encapsulated and then released.

These values are then transformed in relative encapsulation, related to the unitary value defined for the UT samples.

The difference in intracellular delivery efficacy obtained by using the PMMA single channel or the 3D-printed porous cartridge was evaluated.

E. Microscopy optical evaluation of RBC

An inverted fluorescence microscope was used to monitor the eventual morphological changes induced by the encapsulation treatment.

F. *Statistical analysis*

The relative encapsulation outputs were reported in terms of mean and standard deviation. The intracellular delivery relative increase for the stressed sample, in comparison to the UT sample, was statistically compared at each flow rate by using a Paired T-test (p-value threshold 0.001). Moreover, the two sets of results (intracellular delivery obtained through the use of the single channel or the PC) have been statistically compared by using t-tests (p-value threshold 0.001).

III. RESULTS

The results of the encapsulation tests of FD40s into bovine RBCs, suspended in GASP at Ht= 10% were reported. RBCs from the same bovine sample were used for the internal delivery procedure, either by using the standard single-channel PMMA chip (Figure 2) or the porous cartridge at different flow rates (Figure 3). All the treated samples, both in the single channel or in the PC, show a relative encapsulation that statistically differs from the UT sample ($p<0.001$).
The Relative encapsulation obtained with the single channel is statistically superimposable with the one obtained with the PC ($p>0.001$).

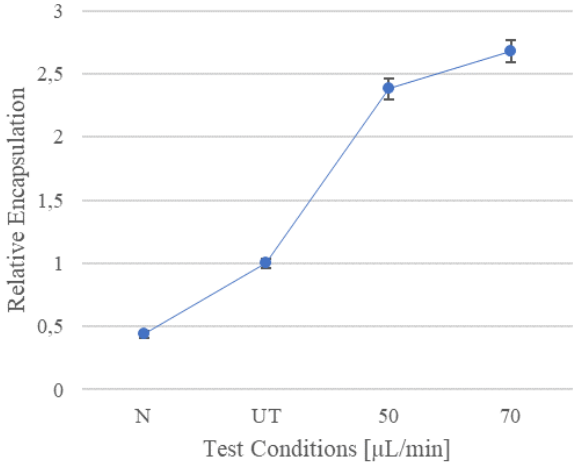

Fig. 2 Evaluation of the encapsulation of FD40 molecules into bovine RBCs after microfluidic shear stresses in the single channel chip. The diagram represents the increase in dextran encapsulation compared to the UT sample, in which the probe molecule enters through spontaneous diffusion.

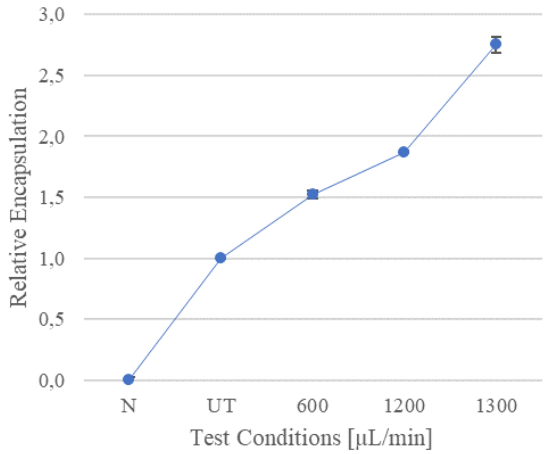

Fig. 3. Evaluation of the encapsulation of FD40 molecules into bovine RBCs after microfluidic shear stresses in the porous device. The diagram represents the increase in dextran encapsulation compared to the UT sample, in which the probe molecule enters through spontaneous diffusion.

Microscopy optical evaluation confirms that RBCs' shape and dimension are preserved either after the passage in the single channel (as already demonstrated in [14] [15]) or the here-tested porous (Figure 4). The treatment does not induce an increase in the number of echinocyte cells ($p<0.001$).

IV. DISCUSSION

It has already been demonstrated that a linear microfluidic channel of appropriate dimensions, when crossed by a fluid suspension of erythrocytes, exploiting controlled shear stress, allows the internal delivery of molecules inside bovine RBCs with good performance.

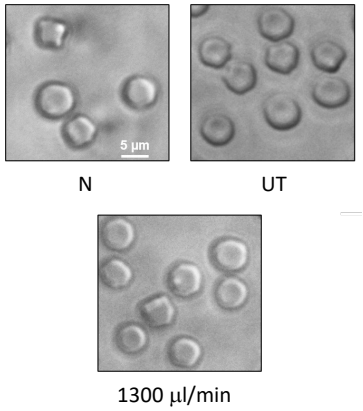

Fig. 4: Picture of bovine RBCs from N and UT samples and after shear solicitation (the example shows the sample stressed at a higher flow rate of 1300 µL/min) through the porous cartridge. Images acquired by inverted microscopy (40X magnification), brightfield, show that the red blood cells do not undergo deformation after the application of microfluidic stresses in the porous device and are not damaged by the presence of dextran.

However, facing the needs of a potential clinical application, this technique has some limitations, such as a low working rate and the need to work at very low dilutions to avoid sedimentation and coagulation of cells in the channel. We, therefore, designed a device that, mimicking the dimensional features of the single fluidic channel, would allow the flow to be parallelized and the rate of internal delivery to be multiplied.
The PC should promote mixing and prevent clotting during the flow procedure. The presented results, obtained by using the prototype of the 3D-printed cartridge for intracellular delivery, confirm these hypotheses. The PC works at higher flow rates, consequently allowing the processing of a higher number of cells if compared to the single channel.
Due to the differences in the bovine erythrocyte membrane characteristics from the human one, the results of the presented tests also act as a proof of concept of the possibility of using the proposed cartridge for the encapsulation process (relative encapsulation always statistically higher than in UT). The transferability of this process to the human RBC has to be verified, as it has already been proven in the single channel [14] [15]. The proposed test aims to directly verify whether the transition from a single channel to a multiple channel leads to a comparable internal delivery result. The results show how the internal delivery efficiency in the new cartridge is comparable in terms of relative encapsulation and confirm that the porous-analog tissue inside the device, generated by the interconnections among the channels, promotes the fluidic cell mixing and favors the internal delivery. At the same time, the

fact that the number of echinocyte cells is comparable with the untreated control allows us to exclude the hypothesis of an effect of the PC material, or better to say that PLA is suitable for contact with RBCs and the buildup of the PC.

All tests are performed at 10% hematocrit, which in previous work [15] was considered a clotting risk condition for the single channel. During the execution of this preliminary test with the new device, however, no clotting problems occurred. This encourages us to continue the development of this intracellular delivery technique, in the direction of increasing the output.

V. Conclusion

In summary, among existing delivery systems, intracellular delivery in different autologous cells is becoming an interesting perspective in many fields, including cancer therapy. When coming to RBCs, their unique membrane properties require a specific combination of applied force and time to avoid hemolysis.

These favorable conditions can be met by using microfluidics, as shown previously. To overcome the previously reported limits, such as the low working rate and the need to use very low dilutions to avoid sedimentation and coagulation of cells in the channel, the here proposed and tested PC, prototyped with 3D printing in biocompatible plastic materials, appears an attainable solution. The experimental tests confirm that the new device promotes the mixing of the cells and the drug, improving the internal delivery and simultaneously limiting the clotting.

The next steps in developing this technique will include a more extensive evaluation of the PC performances, the translatability to the human, and the impact of the intra-patient variability on intracellular delivery efficiency. On the other side, regarding the use of RBCs as drug vectors in cancer therapies, the possibility of using this cartridge to intracellularly deliver compounds of different sizes has to be explored. In fact, in the oncological field, either little molecules, such as chemotherapy drugs, or big molecules, such as monoclonal antibodies, were used.

VI. Acknowledgment

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VII. References

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