Supplementary Information

Influence of plasticisers on pH-dependent drug release and cellular interactions of hydroxypropyl methylcellulose/zein vaginal anti-HIV films containing tenofovir

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S1. SUPPLEMENTARY METHODS

S1.1. Mechanical properties

A TA.TXT*plus* Texture Analyser (Stable Micro Systems, Surrey, UK) on compression mode, with a 30 kg load cell, was used to evaluate the mechanical properties of films. Burst strength and distance at burst were determined using a cylindrical probe (5 mm circular diameter) moving at 0.5 mm/s for puncturing and rupturing a film sample fixed in perpendicular position [1, 2]. Experiments were carried out in quadruplicate for each film. Tensile strength and elongation at break were also determined by using the equipment in tension mode with a 5 kg load cell. Film samples (2 x 4 cm) were placed between tensile grips initially separated by 2.5 cm and moved at 1 mm/s until the film collapsed. Experiments were performed in triplicate, and elongation per area and elastic modulus of the films were calculated.

S1.2. Scanning electron microscopy (SEM)

The surface of films was observed with a scanning electron microscope (JEOL JSM-6335F, JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 15 kV. Films were coated with gold for 90 s in a high vacuum atmosphere. Micrographs were taken at 100x, 500x and 1000x magnifications. Intact and pre-wetted films were observed. In the last case, changes to the microstructure of films were assessed after immersion in SVF for 120 h followed by freeze-drying, as previously described by our group [3]. Freeze-dried samples were then analysed by SEM.

S2. SUPPLEMENTARY RESULTS

S2.1. Drug release – f_2 comparison between different films

Table S1. Similarity factor (f_2) values for the comparison of release profiles obtained for films developed with a single plasticiser ─ polyethylene glycol (PEG), glycerol (G), lactic acid (LA) and oleic acid (OA) ─, in either SVF or SVF/SSF. Values of f_2 lower than 50 are highlighted in bold.

Film comparison		f_2
HZ-PEG – SVF	HZ-G – SVF	**25.7**
HZ-PEG – SVF	HZ-LA – SVF	**21.2**
HZ-PEG – SVF	HZ-OA – SVF	**30.2**
HZ-G – SVF	HZ-LA – SVF	**42.7**
HZ-G – SVF	HZ-OA – SVF	59.6
HZ-LA – SVF	HZ-OA – SVF	**34.8**
HZ-PEG – SVF/SSF	HZ-G – SVF/SSF	**25.3**
HZ-PEG – SVF/SSF	HZ-LA – SVF/SSF	**19.6**
HZ-PEG – SVF/SSF	HZ-OA – SVF/SSF	**15.4**
HZ-G – SVF/SSF	HZ-LA – SVF/SSF	**40.4**
HZ-G – SVF/SSF	HZ-OA – SVF/SSF	**24.1**
HZ-LA – SVF/SSF	HZ-OA – SVF/SSF	**36.2**

Table S2. Similarity factor (f_2) values for the comparison of release profiles obtained for films developed with a blend of plasticisers ─ polyethylene glycol (PEG), glycerol (G), lactic acid (LA) and oleic acid (OA) ─, in either SVF or SVF/SSF. Values of f_2 lower than 50 are highlighted in bold.

Film comparison		f_2
HZ-G/LA – SVF	HZ-G/OA – SVF	71.0
HZ-G/LA – SVF	HZ-PEG/LA – SVF	57.7
HZ-G/LA – SVF	HZ-PEG/OA – SVF	**38.8**
HZ-G/OA – SVF	HZ-PEG/LA – SVF	61.4
HZ-G/OA – SVF	HZ-PEG/OA – SVF	**41.9**
HZ-PEG/LA – SVF	HZ-PEG/OA – SVF	**48.5**
HZ-G/LA – SVF/SSF	HZ-G/OA – SVF/SSF	79.1
HZ-G/LA – SVF/SSF	HZ-PEG/LA – SVF/SSF	52.3
HZ-G/LA – SVF/SSF	HZ-PEG/OA – SVF/SSF	61.6
HZ-G/OA – SVF/SSF	HZ-PEG/LA – SVF/SSF	**48.6**
HZ-G/OA – SVF/SSF	HZ-PEG/OA – SVF/SSF	70.2
HZ-PEG/LA – SVF/SSF	HZ-PEG/OA – SVF/SSF	56.5

Table S3. Similarity factor (f_2) values for the comparison of release profiles obtained for films combining polyethylene glycol (PEG) and oleic acid (OA) at different ratios, in either SVF or SVF/SSF. Values of f_2 lower than 50 are highlighted in bold.

Film comparison		f_2
HZ-PEG1/OA7 – SVF	HZ-PEG1/OA3 – SVF	69.6
HZ-PEG1/OA7 – SVF	HZ-PEG/OA – SVF	73.0
HZ-PEG1/OA7 – SVF	HZ-PEG3/OA1 – SVF	62.6
HZ-PEG1/OA3 – SVF	HZ-PEG/OA – SVF	72.8
HZ-PEG1/OA3 – SVF	HZ-PEG3/OA1 – SVF	76.4
HZ-PEG/OA – SVF	HZ-PEG3/OA1 – SVF	73.1
HZ-PEG1/OA7 – SVF/SSF	HZ-PEG1/OA3 – SVF/SSF	54.8
HZ-PEG1/OA7 – SVF/SSF	HZ-PEG/OA – SVF/SSF	53.1
HZ-PEG1/OA7 – SVF/SSF	HZ-PEG3/OA1 – SVF/SSF	**33.3**
HZ-PEG1/OA3 – SVF/SSF	HZ-PEG/OA – SVF/SSF	81.8
HZ-PEG1/OA3 – SVF/SSF	HZ-PEG3/OA1 – SVF/SSF	**40.3**
HZ-PEG/OA – SVF/SSF	HZ-PEG3/OA1 – SVF/SSF	**48.2**

Table S4. Similarity factor (f_2) values for the comparison of release profiles obtained for films plasticised with polyethylene glycol (PEG) and oleic acid (OA) at ratio 1:7, manufactured with different ratios polymer/plasticiser, in either SVF or SVF/SSF. Values of f_2 lower than 50 are highlighted in bold.

Film comparison		f_2
HZ-PEG1/OA7-20 – SVF	HZ-PEG1/OA7 – SVF	**43.9**
HZ-PEG1/OA7-20 – SVF	HZ-PEG1/OA7-60 – SVF	**25.8**
HZ-PEG1/OA7-20 – SVF	HZ-PEG1/OA7-80 – SVF	**23.6**
HZ-PEG1/OA7 – SVF	HZ-PEG1/OA7-60 – SVF	**35.6**
HZ-PEG1/OA7 – SVF	HZ-PEG1/OA7-80 – SVF	**31.7**
HZ-PEG1/OA7-60 – SVF	HZ-PEG1/OA7-80 – SVF	62.3
HZ-PEG1/OA7-20 – SVF/SSF	HZ-PEG1/OA7 – SVF/SSF	55.6
HZ-PEG1/OA7-20 – SVF/SSF	HZ-PEG1/OA7-60 – SVF/SSF	69.0
HZ-PEG1/OA7-20 – SVF/SSF	HZ-PEG1/OA7-80 – SVF/SSF	66.2
HZ-PEG1/OA7 – SVF/SSF	HZ-PEG1/OA7-60 – SVF/SSF	**37.6**
HZ-PEG1/OA7 – SVF/SSF	HZ-PEG1/OA7-80 – SVF/SSF	**38.7**
HZ-PEG1/OA7-60 – SVF/SSF	HZ-PEG1/OA7-80 – SVF/SSF	82.1

S2.2. SEM micrographs

The surface morphology of films was observed by SEM. Differences among films with a single plasticiser were generally mild (Figure S1). Micropores can be traced in films prepared with glycerol or lactic acid at the solid-state. The presence of micropores may be implicated in facilitated release of TFV from these films. Conversely, films plasticised with PEG or oleic acid featured completely smooth surface, which agrees with the structure observed for zein films by others [4].

After swelling and freeze-drying the surface was eroded and heterogeneous. Films with lactic acid appeared to be more porous, thus supporting the onset of facilitated water diffusion. Less and smaller pores were apparent in films with PEG and glycerol, which ─ as seen in swelling tests (Figure 2 in main text) ─ indicate lower water influx. Finally, films with oleic acid stood out as the most different ones after swelling. Large gaps could be observed, but no micropores, which suggests the unique disposition of zein when plasticised with oleic acid [5]. The presence of hydrophobic groups oriented towards outer layers may limit the formation of micropores when in contact with SVF.

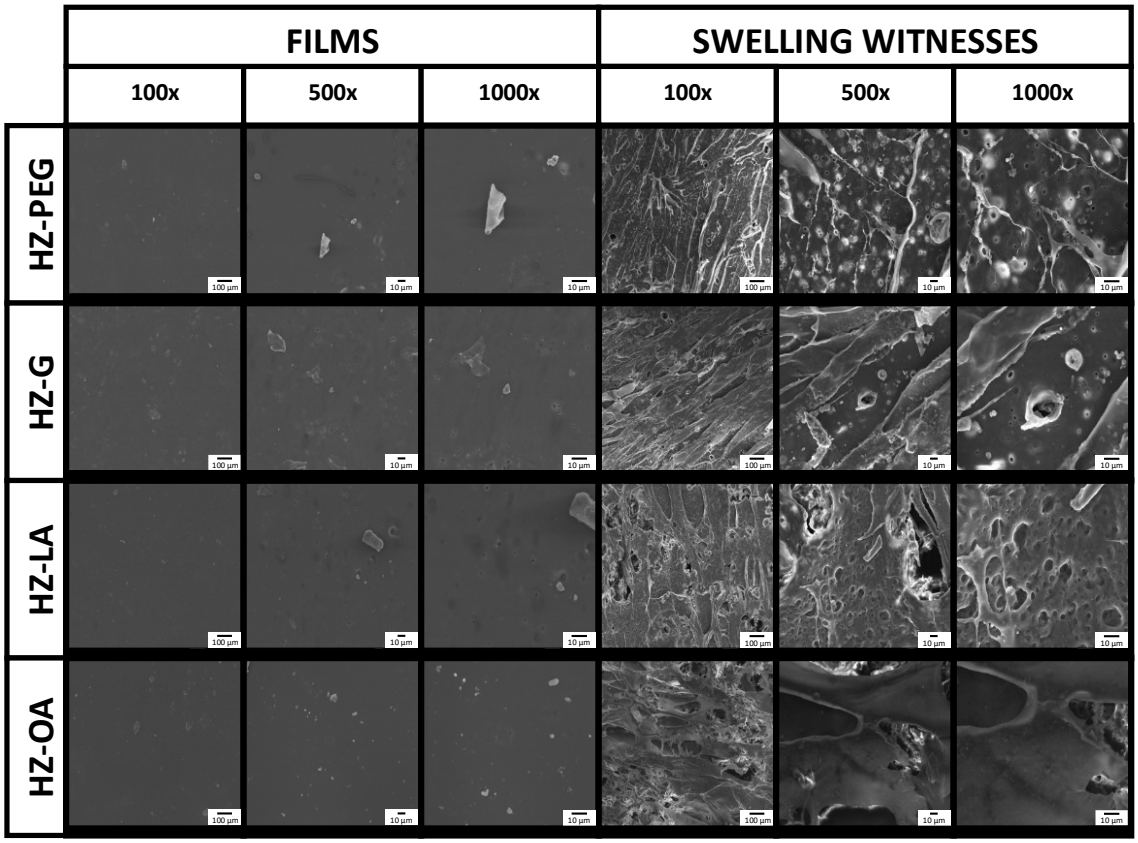

Figure S1. Scanning electron microscopy photographs of films prepared with a single plasticiser ─ polyethylene glycol (PEG), glycerol (G), lactic acid (LA) and oleic acid (OA) ─ at the solid-state (left), and after 120 h immersion in SVF and freeze-drying (right). Representative micrographs were taken with an acceleration voltage of 15 kV and are presented at magnifications of 100x, 500x and 1000x.

Films prepared with a binary mixture of plasticisers featured smooth surface, without the presence of notable micropores (Figure S2). The combination of plasticisers appears to originate a more compact and homogeneous structure, namely when comparing with films prepared with either glycerol or lactic acid. Differences were more apparent after immersion in SVF. Films with glycerol and lactic acid were more porous (pores around 10 μm or smaller), which could help explaining faster drug release. Films with

glycerol/oleic acid and PEG/lactic acid also featured small micropores, although less numerous, as well as aggregates of spherical particles. As observed by other authors, These are in agreement with previous observations of the formation of zein aggregates due to the polymerisation of protein chains [6]. Finally, films with PEG and oleic acid showed large morphological differences, namely heterogeneous surface and big surface gaps. Curiously, these features are similar to those of film prepared only with oleic acid, thus suggesting that this plasticiser is key in defining the structure of films containing PEG and oleic acid.

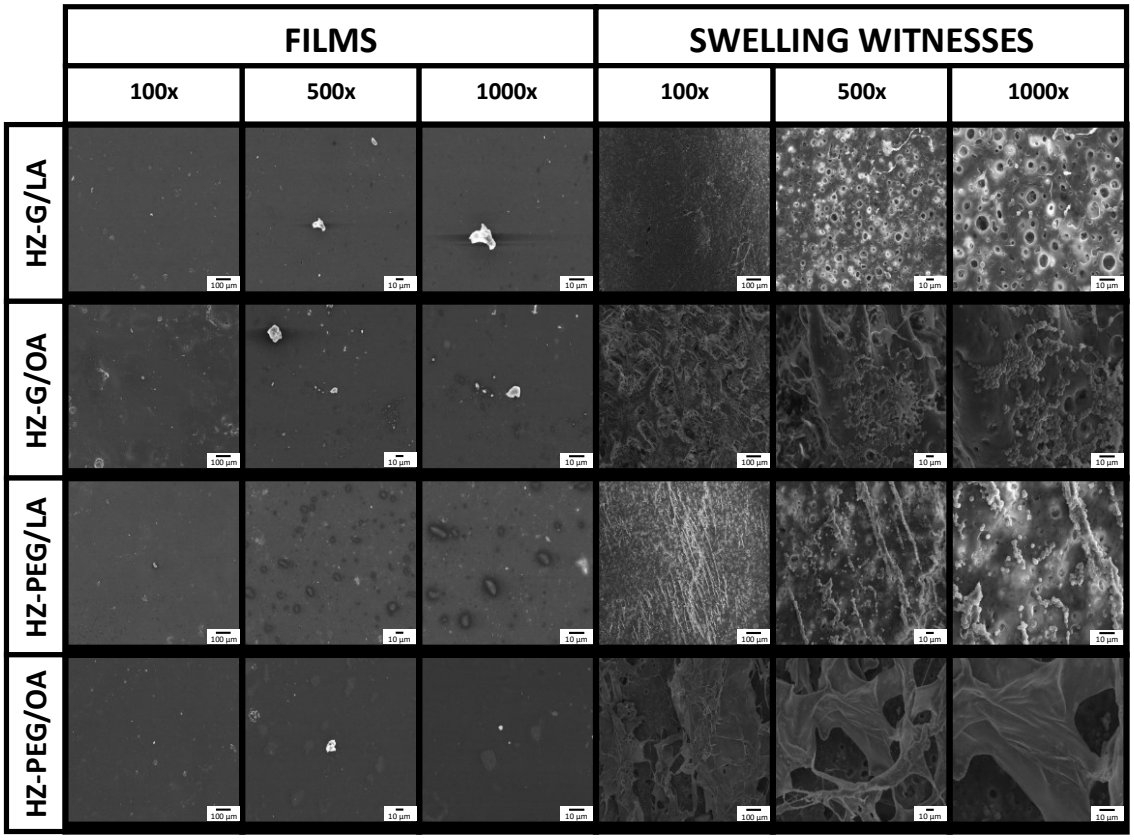

Figure S2. Scanning electron microscopy photographs of films combining two plasticisers ─ polyethylene glycol (PEG), glycerol (G), lactic acid (LA) or oleic acid (OA) ─ at the solid-state (left), and after 120 h immersion in SVF and freeze-drying (right). Representative micrographs were taken with an acceleration voltage of 15 kV and are presented at magnifications of 100x, 500x and 1000x.

Optimised films containing 80% of a binary mixture of plasticisers (PEG/oleic acid at a ratio of 1:7) had a homogenous and smooth surface, with almost no pores being observed before swelling (Figure S3). This agrees with the structure obtained for films combining equal amounts of PEG and oleic acid. The presence of homogeneously distributed pores (10 μm or smaller) was noted after immersion in SVF. These smaller pores (as compared to films prepared with equal amounts of plasticisers) may account for the slower release of TFV from optimised films.

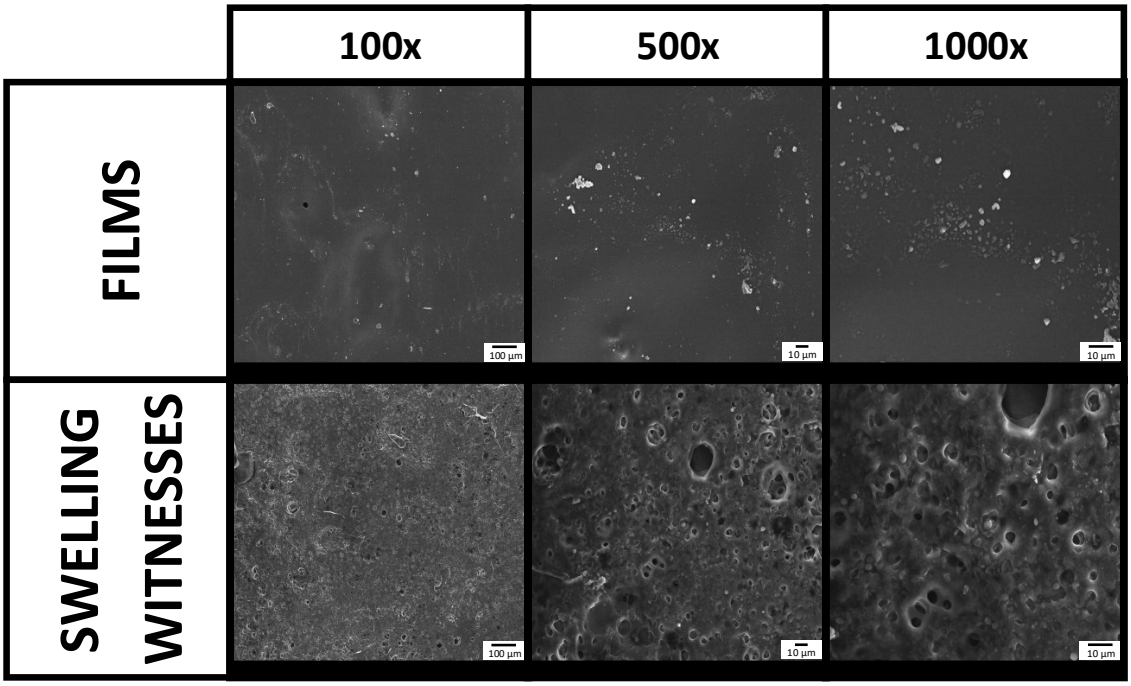

Figure S3. Scanning electron microscopy photographs of films combining 80% plasticisers (PEG and oleic acid at a ratio of 1:7) at the solid-state (top), and after 120 h immersion in SVF and freeze-drying (bottom). Representative micrographs were taken with an acceleration voltage of 15 kV and are presented at magnifications of 100x, 500x and 1000x.

S2.3. Mechanical properties

Films plasticised with lactic acid or PEG featured overall improved mechanical properties as compared to those incorporating either oleic acid or glycerol (Table 3). In particular, these last presented poorer values of resistance and elasticity. Again, the use of binary mixtures of plasticisers appeared to be helpful in providing acceptable mechanical properties to films, and even confirming previous reports of the synergistic interactions of these plasticisers in films containing zein [7]. The formulation optimised regarding drug release presented good mechanical properties, indicating proper plasticisation. The ratio distance/force and the elongation were greater for optimised films and may be contribute to their facile administration and comfortable use [1]. Statistical analysis proved significant differences among films' mechanical properties. Force at burst, and particularly elongation at break, were the parameters most affected as a function of the plasticiser, as shown in multiple comparisons Tukey's test (*Table S6*). The other parameters did not show differences among compared films.

Table S5. Mechanical properties of films prepared with a single plasticiser, prepared with a binary mixture of plasticisers, and optimised regarding drug release (HZ-PEG1/OA7-80), as evaluated using puncture and stretching tests. Results are presented as mean ± SD (*n*=3).

Film	Puncture test		Stretching test			
	Force at burst (N)	**Distance at burst (mm)**	**Tensile strength (N/cm^2)**	**Elongation at break (%)**	**Elongation per area ($\%/cm^2$)**	**Elastic modulus (N/cm^2)**
HZ-PEG	16.03 ± 8.07	2.30 ± 0.08	3.38 ± 0.13	57.1 ± 5.4	7.1 ± 0.7	1.77 ± 0.37
HZ-G	6.37 ± 2.51	1.18 ± 0.10	4.30 ± 0.33	59.2 ± 26.1	7.4 ± 3.3	0.30 ± 0.15
HZ-LA	29.60 ± 9.38	3.54 ± 0.99	1.43 ± 0.26	136.5 ± 11.4	17.1 ± 1.4	0.96 ± 0.17
HZ-OA	5.81 ± 0.89	1.65 ± 0.13	6.61 ± 1.62	48.3 ± 28.5	6.0 ± 3.6	0.73 ± 0.35
HZ-G/LA	29.08 ± 2.45	3.41 ± 0.23	2.58 ± 0.09	144.5 ± 19.7	18.1 ± 2.5	1.27 ± 0.41
HZ-G/OA	8.65 ± 1.28	1.26 ± 0.08	5.66 ± 0.15	80.6 ± 0.3	10.1 ± 0.0	0.38 ± 0.08
HZ-PEG/LA	10.42 ± 2.17	2.25 ± 0.24	2.61 ± 0.43	114.5 ± 14.0	14.3 ± 1.7	1.41 ± 0.22
HZ-PEG/OA	10.37 ± 1.42	1.45 ± 0.26	8.35 ± 0.05	6.7 ± 1.3	0.8 ± 0.2	4.16 ± 0.39
HZ-PEG1/OA7-80	4.41 ± 0.31	1.35 ± 0.16	4.25 ± 0.12	19.1 ± 3.9	2.4 ± 0.5	0.66 ± 0.21

Table S6. p-values obtained in Tukey's multiple comparisons test. Values of $p < 0.05$ (highlighted in bold) were considered as denoting significance.

Film comparison	p-value	
	Force at burst	Elongation at break
HZ-PEG vs. HZ-G	0.4484	>0.9999
HZ-PEG vs. HZ-LA	0.0739	**<0.0001**
HZ-PEG vs. HZ-OA	0.3691	0.7505
HZ-G vs. HZ-LA	**<0.0001**	**<0.0001**
HZ-G vs. HZ-OA	>0.9999	0.4811
HZ-LA vs. HZ-OA	**<0.0001**	**<0.0001**
HZ-PEG vs. HZ-PEG/LA	0.9446	**<0.0001**
HZ-PEG vs. HZ-PEG/OA	0.9417	**<0.0001**
HZ-G vs. HZ-G/LA	**<0.0001**	**<0.0001**
HZ-G vs. HZ-G/OA	0.9999	**0.0022**
HZ-LA vs. HZ-G/LA	>0.9999	0.8354
HZ-LA vs. HZ-PEG/LA	**0.0013**	**0.0014**
HZ-OA vs. HZ-G/OA	0.9994	**<0.0001**
HZ-OA vs. HZ-PEG/OA	0.9841	**<0.0001**
HZ-G/LA vs. HZ-G/OA	**0.0004**	**<0.0001**
HZ-G/LA vs. HZ-PEG/LA	**0.0020**	**<0.0001**
HZ-PEG/LA vs. HZ-PEG/OA	>0.9999	**<0.0001**
HZ-G/OA vs. HZ-PEG/OA	>0.9999	**<0.0001**
HZ-PEG vs. HZ-PEG1/OA7-80	0.2059	**<0.0001**
HZ-OA vs. HZ-PEG1/OA7-80	>0.9999	**<0.0001**
HZ-PEG/OA vs. HZ-PEG1/OA7-80	0.9226	0.3020

S2.4. Cytotoxicity test

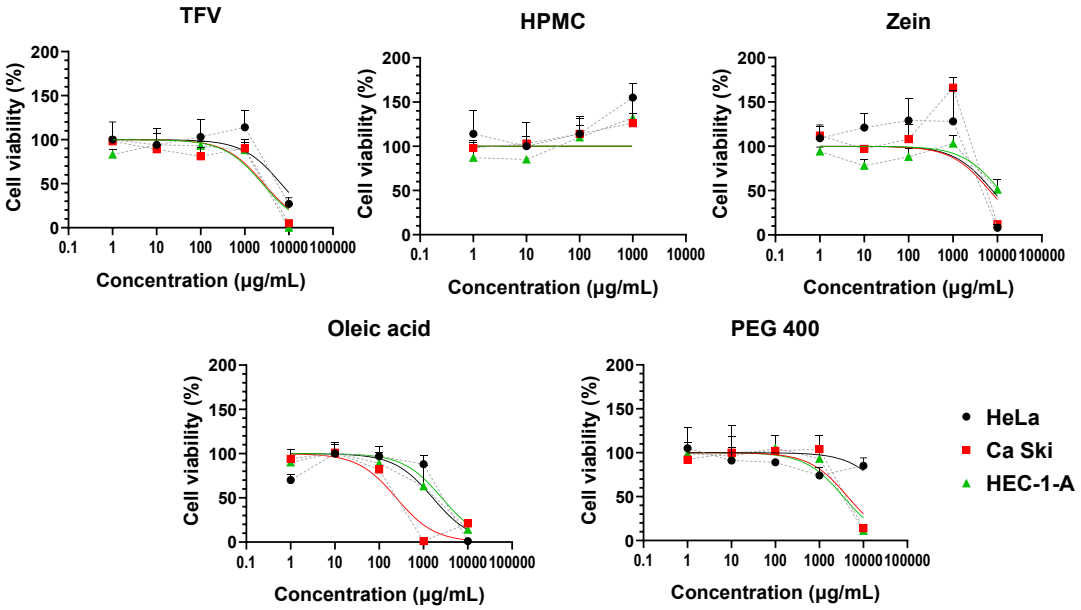

Figure S4. Viability of HeLa, Ca Ski and HEC-1-A cell lines upon exposure to TFV or different excipients, as assessed by the resazurin metabolism assay. Results are presented as mean ± standard deviation (*n*=3). Solid lines represent log-logistic regressions for each plotted data set.

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