Targeting regulation of the tumour microenvironment induces apoptosis of breast cancer cells by an affinity hemoperfusion adsorbent

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ABSTRACT
The cytokine network of tumour microenvironment (TME) plays an important role in cancer growth and progression. The current work aims to provide a new strategy for cancer therapy based on the targeted regulation of cytokines in the TME. Here, heparin-coupled polyvinyl alcohol (PVA-H) microspheres have been developed as an adsorbent for selectively remove tumour-induced immunosuppressive cytokines, such as vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF-β), but not tumour necrosis factor-alpha (TNF-α) which has an immune-stimulating effect and can inhibit tumour growth. The proliferation and apoptosis of breast cancer cells after perfusion were tested by cell viability assays, flow cytometry analysis and mRNA microarray assays. Results showed that the PVA-H microspheres efficiently absorbed the majority of VEGF (74.39%) and TGF-β (86.39%), but much less TNF-α (4.16%). The regulation of the cytokines had remarkable anti-proliferative and pro-apoptotic effects on breast cancer cells, which was further confirmed from the change of mRNA expression levels. Thus, targeting regulatory pathways within the TME by an affinity adsorbent that selectively depletes immunosuppressive cytokines is potentially a new and promising strategy for cancer therapy.

Introduction
The tumour microenvironment (TME) plays an essential role in the occurrence, development and metastasis of cancer [1]. In the TME, plenty of cytokines account for tumour progression, recruitment of tumour-associated immune cells, and immunosuppressive effects. Therefore, regulation of these cytokines can reverse the immunosuppressive microenvironment and improve anti-tumour efficiency. These tumours are hypervascularized because of the overexpression of proangiogenic factors [2], such as tumour-induced immunosuppressive cytokines (e.g. vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF-β)), especially in breast cancer. VEGF is vital for the direct regulation of the cell survival and growth of breast cancer cells and blocking VEGF has direct and rapid antiangiogenic effects in the tumour [3,4]. TGF-β has a strongly pro-tumour effect at later stages of cancer [5]. Nancy Dumont et al. [5] had obtained the data relating to the cellular and molecular mechanisms by which TGF-β signalling can accelerate the natural history of tumour development. Whereas tumour necrosis factor-alpha (TNF-α) is one of the strongest anti-cancer factors that is frequently upregulated in various types of cancer, which can enhance an antitumor immune response in the TME. For clinical applications, TNF-α is safe and high efficient for the treatment of soft tissue sarcoma [6]. In addition, other immunosuppressive cytokines (IL-4 and IL-10) and immune-stimulating cytokines (IL-1β and IL-6) can also regulate the proliferation and apoptosis of tumour cells [7]. In response to TME, eliminating the excess tumour-promoting cytokines shown obvious advantages in overcoming tumour proliferation and hypervascularity [8].

For instance, Christopher G Willett et al. [9] used bevacizumab to cure rectal cancer patients and indicated that VEGF blockade had a direct and rapid antivascular effect in human tumours. Jianming Xu et al. [2] developed a novel small-molecule inhibitor (Surufatinib) that targets proangiogenic factors, which could reduce tumour angiogenesis and enhance antitumor activity. However, the cost of these drugs treatment is high, and it also has clinically obvious pharmacodynamic limitations in respect of hypertension and proteinuria. Beyond conventional drug administration, hemoperfusion has been proved to be an effective and safe therapy to eliminate toxins in blood and is very successfully used in clinical. Yoshiki Yamamoto et al. [10] reported extra-fine fibres for
efficiently TGF-β removal and prolonged a longer survival time of the tumor-bearing rats through hemoperfusion. Therefore, it is of great clinical significance to develop a novel selectively adsorbent with higher adsorption capacity, aiming at simultaneous tumour-induced immunosuppressive cytokines (VEGF and TGF-β) cleansing, without a significant reduction of immune-stimulating cytokine (TNF-α) in a hemoperfusion system, to offer another effective technique for the regulation of TME in cancer treatment.

Polyvinyl alcohol (PVA) microspheres have been intensively investigated as an ideal carrier [11], owing to their favourable mechanical properties, abundant macropore structure, and excellent biocompatibility, which are very important for hemoperfusion. Furthermore, a large number of hydroxyl groups on PVA can be easily functionalized by non-covalent and covalent chemical interactions. It is also widely reported that PVA beads represent a type of anti-fouling polymer, and have few adverse effects on blood components [12]. Heparin is a highly sulphated linear polysaccharide belonging to the family of glycosaminoglycans and has been widely used as an anticoagulant agent. In the field of adsorbent-based hemoperfusion, heparin is always introduced to functionalize polymers in order to improve the blood compatibility of adsorbent, or used as a good ligand for the removal of certain toxins, including endotoxin and low-density lipoprotein (LDL) [13]. However, the role of PVA-H in targeting regulation of TME using hemoperfusion column is not reported. To the best of our knowledge, VEGF contains some high-affinity binding sites for heparin, and therefore the heparin-functionalized adsorbent could be a good choice for the regulation of the TME through efficiently eliminating the excess VEGF [14]. Moreover, heparin and TNF-α (pI = 5.6) molecules are negatively charged, while VEGF (pI = 8.5) and TGF-β (pI = 8.9) molecules are positively charged [15]. Therefore, heparin would be expected to selectively bind with VEGF and TGF-β by electrostatic interaction, but for TNF-α, this binding ability should be weak.

In the present study, immobilized heparin onto PVA beads (PVA-H) were prepared, which was demonstrated that targeting regulation of cytokines in the TME by decreasing the levels of VEGF and TGF-β had anti-proliferative and pro-apoptotic effects on breast cancer cells (Scheme 1), offering a potential new strategy for breast cancer therapy.

Materials and methods

Immobilisation and characterisation of PVA and PVA-H microspheres

Polyvinyl acetate (PVA) microspheres were synthesized using a suspension polymerization method [16] and the PVA-H microspheres were formed through the crosslinking between PVA beads and heparin (refer to Supplementary Information for details of synthetic strategy). Furthermore, the detailed characterization was provided in the Supplementary Information.

Adsorption experiments

For testing the adsorption performance of these PVA-H microspheres for VEGF, TGF-β, and TNF-α, human plasma was spiked with the recombinant human cytokine at a target concentration of 1000 pg/mL. Prior to use for adsorption experiments, the microspheres were allowed to swell fully in 0.9% saline solution. For brevity, details were provided in the Supplementary Information.

Regulation of cytokine network of TME by PVA-H microspheres

Cell culture

SHZ-88 rat breast cancer cells were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, they cultured in complete RPMI 1640 media at 37 °C in an atmosphere of 5% CO₂. Cell lines were subcultured for the subsequent experiment by adding 0.25% trypsin, 10% FBS, and fresh RPMI 1640 media. All of the cell culture materials were supplied by Biological Industries Israel Beit Haemek Ltd.

Scheme 1. Schematic illustration of targeting regulatory pathways within the TME induces apoptosis of breast cancer cells by an affinity adsorbent which can selectively deplete immunosuppressive cytokines.
Cell viability assay
The cells were seeded into 96-well plates at a density of 3000 cells per well and cultured in their medium (refer to Supplementary Information for details of the preparation of medium) respectively. The cell viability was assayed with the CCK-8 Kit according to the manufacturer’s instructions. CCK-8 was detected at 0, 6, 12, 24, 48 and 72 h. Each experiment was done in sextuplicate.

Annexin V–FITC and PI assay
The cells were seeded into 6-well plates at a density of $9 \times 10^4$ cells per well and cultured in their medium respectively. Cell apoptosis at 48 and 72 h were assessed by using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, USA) according to the manufacturer’s instructions, and the stained cells were analyzed by two-color flow cytometry.

RNA preparation and mRNA microarray experiments
Gene expression at 72 h was measured in mRNA microarray assays, which were performed by the Cnkingbio Biotechnology Corporation (Beijing, China). Total RNA was extracted from cells using the TRIzol reagent (Invitrogen, Carlsbad, Canada) and purified with an RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA integrity and purity were investigated by absorption spectroscopy and electrophoresis. The hybrid chip was scanned using an Affymetrix Scanner 3000 7G laser confocal scanner (Affymetrix, USA), and then the bioinformatics analysis was performed.

Statistical analysis
All results are presented as mean ± SD, as noted in the text. Two-tailed non-paired t-test was used to compare the difference between two groups in GraphPad Prism (GraphPad Software Inc.), and statistical significance was displayed as * when $p < .05$, ** when $p < .01$, **** when $p < .001$, ***** when $p < .0001$ and "ns" when $p > .05$.

Results and discussion
Characterisation
Scanning electron microscope (SEM) images (Figure 1(a)) showed that both PVA and PVA-H possessed a well-formed...
spherical shape with a mean diameter of 100 µm and a highly porous structure. The results indicated that the functionalization of heparin by means of employing glutaraldehyde as a spacer arm did not have any significant impact on the structure of the microspheres, and clear macropores could still be seen on their rough external surfaces.

A fluorescence labelling strategy was also used to provide more direct evidence. These special PVA-H microspheres were prepared using fluorescein isothiocyanate (FITC)-labeled heparin (ThermoFisher, #H7482). Figure 1(b) shows the images of FITC-heparin immobilized PVA compared with unmodified PVA beads, an obvious fluorescence signal was detected on the PVA-H, while no signal was detected for the original PVA beads. Therefore, the presence of heparin on the PVA-H microspheres could be confirmed [17].

The nature of the functional groups of the PVA and PVA-H microspheres was characterized by FTIR spectroscopy, as shown in Figure S1. After the functionalization of heparin, the PVA peak at 1124 cm⁻¹/C₀ was shifted to 1091 cm⁻¹/C₀, probably due to the overlapping of the heparin peak at 1020 cm⁻¹/C₀ (sulphonic acid group). This indicates that heparin was present in the PVA-H microspheres [18]. X-ray photoelectron spectroscopy (XPS) analyses of the obtained materials were also conducted to characterize the heparin component (SO₃²⁻) on the surface of the PVA-H microspheres. As shown in Figure 1(c) and Table S1, the surface sulphur content of

Figure 2. The adsorption characteristics of PVA-H for VEGF, TGF-β and TNF-α in human plasma. (a) Non-linear fitting for the adsorption kinetic data of VEGF onto the two adsorbents in human plasma (T = 37 °C, Cلاقة = 1335.21 ± 3.92 pg/mL). (b) Effects of temperature on adsorption of VEGF (t = 2 h, CVEGF = 1152.32 ± 4.23 pg/mL). (c) Non-linear fitting for the adsorption kinetic data of TGF-β onto the two adsorbents in human plasma (T = 37 °C, CTGF-β = 1048 ± 1.24 pg/mL). (d) Effects of temperature on adsorption of TGF-β (t = 2 h, CTGF-β = 1121 ± 2.34 pg/mL). (e) Non-linear fitting for the adsorption kinetic data of TNF-α onto the two adsorbents in human plasma (T = 37 °C, CTNF-α = 1069 ± 3.24 pg/mL). (f) Effects of temperature on adsorption of TNF-α (t = 2 h, CTNF-α = 968 ± 4.25 pg/mL). The plasma to adsorbent ratio was 20 and all values are expressed as mean ± SD (n = 3). ns: not significant, **p < .01, ***p < .001, ****p < .0001.
the PVA-H microspheres was about 0.07 wt.%, and the sulphur element could be detected at 163.7 eV. For comparison, PVA microspheres had no sulphur elements as is shown in Figure S2. These results demonstrated that heparin was successfully immobilized on the PVA microspheres.

Assessment of adsorption characteristics

The PVA-H microspheres were prepared using different amounts of heparin. As shown in Figure S3, the optimal dosage of heparin was identified as 60 mg in order to achieve the best adsorption capacity of the PVA-H microspheres for VEGF (70.37%, 31.31 ng/g). Next, the adsorption characteristics of PVA-H for VEGF, TGF-β, and TNF-α in human plasma were compared, as shown in Figure 2, Figure S4 and Table S2. The results of time-dependent adsorption kinetics (Figure 2(a)) demonstrated that the uptake of VEGF by PVA-H was quite rapid at the very beginning and reached equilibrium within 90 min. The final VEGF adsorption rate of PVA-H reached 71.23%, with an adsorption capacity measured as 32.06 ng/g. In contrast, the comparable VEGF adsorption rate of PVA alone was only about 5%, which was clearly much lower than that of the PVA-H microspheres. Furthermore, the kinetic data were analyzed using two extensively used kinetic models (pseudo-first-order and pseudo-second-order models). The nonlinear form of the two models is expressed as equations:

\[
\ln \left( \frac{Q_e}{Q_t} \right) = k_1 t \\
Q_t = \left( k_2 Q_e^2 t \right) / \left( 1 + k_2 Q_e^2 t \right)
\]

where, \(k_1 (\text{min}^{-1})\) is the first-order equilibrium rate constant, \(k_2 (\text{g/ng min})\) is the second-order equilibrium rate constant, \(Q_e\) and \(Q_t\) are the adsorption quantity (ng/g) at equilibrium and at time \(t\), respectively. The relevant parameters (\(Q_e, exp\), \(Q_e, calc\), \(k\)), the correlation coefficients (\(R^2\)), and non-linear fitting are shown in Table S2 and Figure 2(a,c,e). It could be seen that \(R_1^2\) was higher than \(R_2^2\) value, which was manifested that the physical adsorption played an important role in the adsorption of VEGF by PVA-H (Table S2 and Figure 2(a)) [19]. In addition, as shown in Figure 2(c), PVA-H also had an excellent adsorption capacity for TGF-β (86.39%, 30.28 ng/g) compared with PVA (62.85%, 20.58 ng/g), and was able to reach adsorption equilibrium quickly. From the data of TGF-β onto the two adsorbents (Table S2), pseudo-second-order had a higher \(R_2^2\), indicating the adsorption of TGF-β using PVA-H was dominated by the chemical adsorption [20]. Even more noteworthy is the fact that over time,
PVA-H barely adsorbed any TNF-\(\alpha\) (less than 4.5%), a representative of the immune-stimulating cytokines, which is shown in Figure 2(e). It is precisely because the heparin is a linear sulphated polysaccharide, molecules of which are negatively charged, which is similar to TNF-\(\alpha\) (pi ~ 5.6). In contrast, VEGF (pi ~ 8.5) and TGF-\(\beta\) (pi ~ 8.9) molecules are positively charged. Therefore, PVA-H can selectively bind with VEGF and TGF-\(\beta\) but not TNF-\(\alpha\) through electrostatic interactions between the sulphonic acid groups of heparin and the basic amino acids of cytokines. Taken together, the adsorption characteristics of PVA-H microspheres for cytokines had a certain selectivity, which was beneficial for the following regulation of the TME by removing immunosuppressive but not immunostimulatory cytokines.

The effect of temperature on cytokine adsorption is shown in Figure 2(b,d,f). The degree of cytokines adsorption on PVA-H significantly increased with the increase of temperature, probably due to the conformational transition leading to the increased surface hydrophobicity of cytokines at higher temperatures [19,21]. However, adsorption is an exothermic process, such that after a certain temperature was reached, the binding affinity decreased with increasing temperature.

**Regulation of cytokine network of TME by PVA-H microspheres**

Before this experiment, we had established that cultures of SHZ-88 cells were minimally affected by exposure to a cell culture medium that had been perfused through the PVA-H microspheres (Figure S6). Experiments to determine the effects of PVA-H microspheres on the regulation of the TME could then be performed (Figure 3(a)). During the attachment of breast cancer cells for 12 h, abundant VEGF and TGF-\(\beta\) were produced and secreted into the culture medium, whereas the amount of TNF-\(\alpha\) was negligible (less than 5 pg/mL). Meanwhile, the expression of IL-1\(\beta\), IL-4, IL-6, and IL-10 in the cell culture supernatants were measured using a pre-mixed multi-analyte kit (R&D Systems), whereas the concentrations were too low to detect (data not shown). In addition, VEGF and TGF-\(\beta\) are generally regarded as two of the most representative immunosuppressive cytokines during the growth and metastasis of tumor, and their overexpression makes an inactive effect for the cure of cancer, while TNF-\(\alpha\) is a typical proinflammatory cytokine. Therefore, VEGF, TGF-\(\beta\), and TNF-\(\alpha\) were chosen to assess the perfusion capacity.

After perfusing these supernatants through the PVA-H microsphere column, as shown in Figure 3(b), the concentration of VEGF therein sharply decreased from 490 to 92 pg/mL, and the concentration of TGF-\(\beta\) also decreased significantly from 345 to 121 pg/mL. Then, HUVECs were cultured in the conditioned medium with or without PVA-H perfusion for tube formation assay. The results demonstrated the perfusion using PVA-H microspheres can restrain the tube formation (Figure S7). Next, the effect of perfusion on the viability of SHZ-88 cells were assessed using the Cell Count Kit-8 (CCK-8) assay. As shown in Figure 3(c), the cell viability of the treated group was lower than that of the control group at all different time points (0, 6, 12, 24, 48 and 72 h). This model of regulation in the TME indicates that the cell proliferation of...
SHZ-88 was dramatically inhibited when these cytokines were removed. Moreover, Figure S8 showed the proliferation of rat breast cancer cells was promoted by adding VEGF and TGF-β back into the treated medium at 24 and 48 h (add back group), which demonstrated that the VEGF and TGF-β played a vital role in the proliferation of SHZ-88 cells.

Flow cytometry analysis with Annexin V and propidium iodide was also performed to measure apoptosis of the SHZ-88 breast cancer cells. In Figure 4, the flow histogram quadrants Q1, Q2, Q3, and Q4 indicate necrotic cells, late-stage apoptotic cells, early-stage apoptotic cells, and normal cells, respectively. The proportions of apoptotic cells in the treated group at 48 and 72 h reached as much as 47.69 and 44.6%, respectively. For the untreated group, the proportions of apoptotic cells at 48 and 72 h were only 15.17 and 22.74%, respectively. Altogether, it is concluded that the cell apoptosis of SHZ-88 was promoted in this model of regulating the TME by depleting anti-inflammatory cytokines.

Therefore, these results demonstrated that the regulation of the TME using adsorbent-based hemoperfusion in this model had marked anti-proliferative and pro-apoptotic effects on breast cancer cells due to the removal of excess immunosuppressive cytokines including VEGF and TGF-β. We thus envisioned our hemoperfusion adsorbent as an ideal candidate for cancer treatment.

In order to evaluate the effect of perfusion at the level of gene expression of SHZ-88 cells, mRNA microarray assays were performed after 72 h exposure. As shown in Figure S9, we identified 954 significantly differentially expressed genes by setting the absolute fold change threshold to >1.2 and the p-value at <.05. It can be seen from the cluster gram that the samples from the two groups are clearly separated, which indicates a robust response to PVA-H perfusion treatment. Among these 954 differentially expressed genes, 420 genes were downregulated and 534 genes were upregulated. It is worth noting that in Figure S10, we had further chosen to focus on genes involved in proliferation and apoptosis. Figure 5(a), Tables S5 and S6 showed the genes related to positive and negative regulation of proliferative and apoptotic functions (Left: proliferation-related genes; Right: apoptosis-related genes).

Figure 5. Mechanism of the anti-proliferative and pro-apoptotic effects on breast cancer cells. SHZ-88 cells were cultured for 12 h and then exposed to supernatants treated or not treated by perfusion through PVA-H microsphere columns. (n = 4). (a) Genes involved in positive and negative regulation from the genes involved in proliferation and apoptosis. (Left: Genes associated with positive and negative regulation of cell proliferation; Right: Genes involved in positive and negative regulation of apoptosis). (b) Functionally significantly related genes involved in the proliferative and apoptotic functions. (Left: proliferation-related genes; Right: apoptosis-related genes).

Figure 6. KEGG pathway enrichment analysis of the differentially expressed genes (Reds are up-regulated, petunias are down-regulated).
down-regulated (light blue), while those negatively regulating proliferation were up-regulated (red). Genes positively regulating apoptosis were up-regulated (red), while those negatively regulating apoptosis were down-regulated (light blue). Thus, as for the functionally significantly related genes involved in the proliferative and apoptotic functions (Figure 5(b)), on the basis of related research, not only the down-regulated genes, such as gene Timp3, Ppargc1a, Lep and Nck2, but also the up-regulated genes, such as gene Blm, Rag1, Lifr and Ptk2b, would promote apoptosis of breast cancer cells [22–31]. More importantly, both the down-regulated genes, such as gene Mmp12, Cxcl1, Lep and Wnt10b, and the up-regulated genes, such as gene Gpc3, Miip, Ovol1 and Fhl1, would inhibit the proliferation of breast cancer cells [27,28,32–41]. Taken together, these results imply that most genes involved in proliferation and apoptosis were changed in such a manner that they exhibited a positive tendency to inhibit proliferation and promote apoptosis of the breast cancer cells after treatment by the perfusion through PVA-H microspheres.

To gain a better understanding of how the perfusion through PVA-H microsphere columns affects the tumour, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed as shown in Figure 6. Notably, the TGF-beta signalling pathway was clearly enriched in those pathways that were down-regulated after treatment, which was consistent with the previous results. Next, gene ontology (GO) enrichment analysis showed that the antitumor activities had been up-regulated, such as the negative regulation of the vascular endothelial growth factor signalling pathway, positive regulation of cytokine production involved in inflammatory response, regulation of macrophage chemotaxis, regulation of chromatin silencing, long-chain fatty acid import, and others (Figure 7). In contrast, the pro-tumour activities had been down-regulated, for instance, the inactivation of MAPK activity, negative regulation of Ras protein signal transduction, DNA integration, fructose transport, and others. KEGG pathway and GO enrichment analysis were combined to demonstrate that both VEGF and TGF-β were down-regulated at the gene level. It was concluded that this study provided a facile strategy to fabricate a selectively affinity adsorbent by hemoperfusion for cancer therapy.

**Blood compatibility**

Haemolysis experiments, blood routine tests, platelet adhesion and coagulation assays were conducted to evaluate the blood compatibility of the PVA-H microspheres (refer to Supplementary Information). For the haemolysis assay, the mean optical density (OD) values of the negative and positive controls were 0.0191 ± 0.0051 and 0.8915 ± 0.0047, respectively, with both these values conforming to the ISO 19003-
4:2002(E) standard. As shown in Figure 8(b) and Table S3, the haemolysis ratio for PVA-H microspheres was about 3.22 ± 0.24% (<5%), indicating that the functionalization of heparin on the PVA microspheres exhibited little risk of facilitating haemolysis during application. The influence of PVA-H microspheres on blood components after 60 min of direct contact is also shown in Table S3. It was found that PVA-H microspheres had negligible negative effects on the majority of blood cells including white blood cells (WBC) and red blood cells (RBC), as compared with the normal group. A little platelet (PLT) adsorption was observed for PVA and PVA-H microspheres, which was still in an acceptable range for clinical uses (<20%) (Figure 8(a)). Therefore, these results demonstrated that PVA-H microspheres possessed an excellent blood compatibility profile and had a high potential to be applied in hemoperfusion for cancer treatment.

Conclusions
In summary, heparin was successfully immobilized onto PVA microspheres as an adsorbent for selectively removing tumour-induced immunosuppression cytokines (VEGF and TGF-β) but to retain the anti-tumour cytokines (TNF-α) in the TME by perfusion. The proliferation of the rat breast cancer cells was inhibited, and apoptosis was promoted. mRNA microarray assays indicated that differentially expressed genes in treated cells were involved in inhibiting proliferation and promoting apoptosis of the breast cancer cells. Furthermore, KEGG pathway and GO enrichment analysis were combined to demonstrate that both VEGF and TGF-beta were down-regulated at the gene level. Besides, the PVA-H microspheres had excellent blood compatibility (a negligible haemolytic activity and platelet adhesion) for hemoperfusion.

Compared to antibody-based drugs, hemoperfusion is a safer and more economical therapy, which has been widely used in the treatment of acute drug poisoning, liver failure and other difficult miscellaneous diseases. Therefore, hemoperfusion with PVA-H microspheres targeting the regulation of TME may be a useful and cost-effective approach for cancer treatment.

Supplementary data related to this article can be found on the publisher’s website.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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Data availability statement
The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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