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ORIGINAL RESEARCH PAPER

A novel 'pipeline' system for downstream preparation of therapeutic monoclonal antibodies

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Abstract A novel 'pipeline' system for the preparation of therapeutic monoclonal antibodies (mAb) in a non-GMP compliant environment has been developed. We used sterile silica-gel pipes to connect individual process units, in order to form a fully-enclosed and seamlessly connected system. This 'pipeline' system was used to implement downstream preparation processes for a humanized anti-CD146 mAb, huAA98, which is a therapeutic mAb generated to inhibit cancer-related angiogenesis. The quality assessment of the huAA98 end-product indicated that endotoxin levels were 0.016 EU/ml, protein A levels were 1.08 ng/ml and host cell protein (HCP) was undetectable. Thus, all measures were below the clinical criteria set by the Chinese Pharmacopoeia (Edition 2010). Having passed our proof-of-concept test, this 'pipeline' system can be used as a universal platform for the preparation of mAbs suitable for pre-clinical studies, in a non-GMP compliant laboratory environment.

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Introduction

Therapeutic mAbs currently dominate the biologics market (Reichert 2012; Li and Zhu 2010), and more efficient mAb drugs are in ever-increasing demand. For early stage drug development, large-scale manufacturing of clinical-grade mAbs presents a bottleneck (Munro et al. 2011; Horenstein et al. 2003). Due to the open operation manner of downstream preparation steps, the conventional resolution is to build Good Manufacture Practice (GMP) compliant facilities to obtain clinical-grade mAbs (Gottschalk 2008).

Here, we propose a 'pipeline' system as a cost-efficient supplement to GMP-compliant facilities for early-stage development of mAb drugs. We introduce a novel approach to downstream preparation with all processes executed in a sterile and fully-enclosed environment, like a 'scale-down' GMP version. Importantly, the modular design of our system allows for a high degree of flexibility, to accommodate for modifications in composition and sequence of processing modules, as would be required for the preparation of different mAbs.

In general, downstream preparation of mAbs takes place in three phases: a clarification step, 2–3 chromatography steps, and a formulation step. In

compliance with these three general phases, we designed three types of fully-enclosed preparation modules: a micro-filtration module, a chromatography module, and an ultra-filtration module, with all modules connected by sterile silica-gel pipes to form an intact 'pipeline' system. In practice, the types and quantity of modules, as well as their sequence can be altered according to the preparation procedure chosen.

As a proof-of-concept for our 'pipeline' system, we completed a preparation of the huAA98, which is a humanized version of an anti-CD146 mAb, the murine version of which inhibits angiogenesis and suppresses growth of various tumors (Yan et al. 2003; Wang and Yan 2013). We provide evidence that our 'pipeline' system allows for high production purity and ensures biological activity of the end-product.

Materials and methods

Instrumentation and materials

Storage bags, connectors, micro-filtration hollow fibre columns, ultra-filtration hollow fibre columns, the chromatography system, chromatography media and chromatography columns were purchased from a commercial supplier (GE Healthcare). Silica-gel pipes and peristaltic pumps were purchased from Longer Precision Pump Co., Ltd. All solutions were prepared using clinical-grade water.

General procedures

All product-contact surfaces were exposed to 0.1 M NaOH for at least 2 h, and subsequently rinsed with clinical-grade water. Endotoxin residues in rinse sample were no higher than 0.3 EU/ml. All buffers were filtered through 0.2 μm membrane filters into sterilized containers and stored at room temperature.

Downstream preparation processes of huAA98

Clarification of the huAA98 supernatant

The huAA98 supernatant was filtred via the micro-filtration module at a circular flow rate of 2,000–4,000 ml/min, while the clarified supernatant was collected for subsequent chromatographic purification.

Affinity chromatography capture of huAA98

An XK 50/20 column with MabSelect SuRe media was equilibrated with binding buffer (50 mM sodium phosphate, 50 mM NaCl, pH 7.2), before the clarified supernatant was loaded onto a column, with a residence time of 4 min. After washing with binding buffer, a step elution was performed using elution buffer (20 mM sodium citrate, pH 3.0), which was followed by neutralization with 0.15 ml of 1 M Tris/HCl (pH 9.5) per 1 ml eluate, which was injected into the collection pool by a sterile syringe.

Gel filtration chromatographic purification of huAA98

The XK 50/100 column with Superdex-200 media was equilibrated with phase buffer [20 mM sodium phosphate, 10 % (v/v) glycerol, pH 6.4], prior to the sample loading at a 30 cm/h. The loading volume was less than 2.5 % of the column volume. Two peaks were identified after separation with a Superdex-200 column, and the second peak was collected for further processing.

Concentration and formulation process

The solution collected through the gel filtration chromatography was concentrated by the ultra-filtration module at 1,000–2,000 ml/min. After ten-fold concentration of the mAb, tenfold volumes of storage buffer [50 mM sodium phosphate, 10 % (v/v) glycerol, pH 6.4] were fed into the system with multiple repeats of the concentration step. Subsequently, the huAA98 end-product was transferred into sterile, fully-enclosed bottles and stored at 4–80 °C until further use.

Analytical quality control of huAA98

Protein concentrations were determined from the absorbancy at 280 nm. An absorbance coefficient of 1.641 AU per mg of total huAA98 protein was used. A chromogenic substrate method was used to determine endotoxin levels, as described in appendix XII E of "Chinese pharmacopoeia" (Edition 2010). A protein A ELISA kit was used to determine protein A residues in the mAb preparation. A CHO HCP detection kit was used to determine the presence of HCP residues. Reducing/non-reducing 12 % SDS-PAGE was used in

combination with SEC-HPLC to analyze the protein purity of the mAb preparation.

Analysis of biological activity of the huAA98 end-product

To determine the binding activity of huAA98 to CD146, a western blot and a whole cell ELISA were conducted as described previously (Yan et al. 2003; Zhang et al. 2008). In order to test the inhibitory activity of huAA98, a tube formation assay and a migration assay were executed as described by us earlier (Yan et al. 2003; Zheng et al. 2009).

Results and discussion

Construction of our 'pipeline' system

We designed three types of fully-enclosed preparation modules. The results for the construction of each module are described below.

To construct the micro-filtration module, a micro-filtration hollow fibre column, a storage bag as a circulation pool, a storage bag as a feed pool, a storage bag as a collection pool and two peristaltic pumps were connected by silica-gel pipes in a manner as shown in Fig. 1.

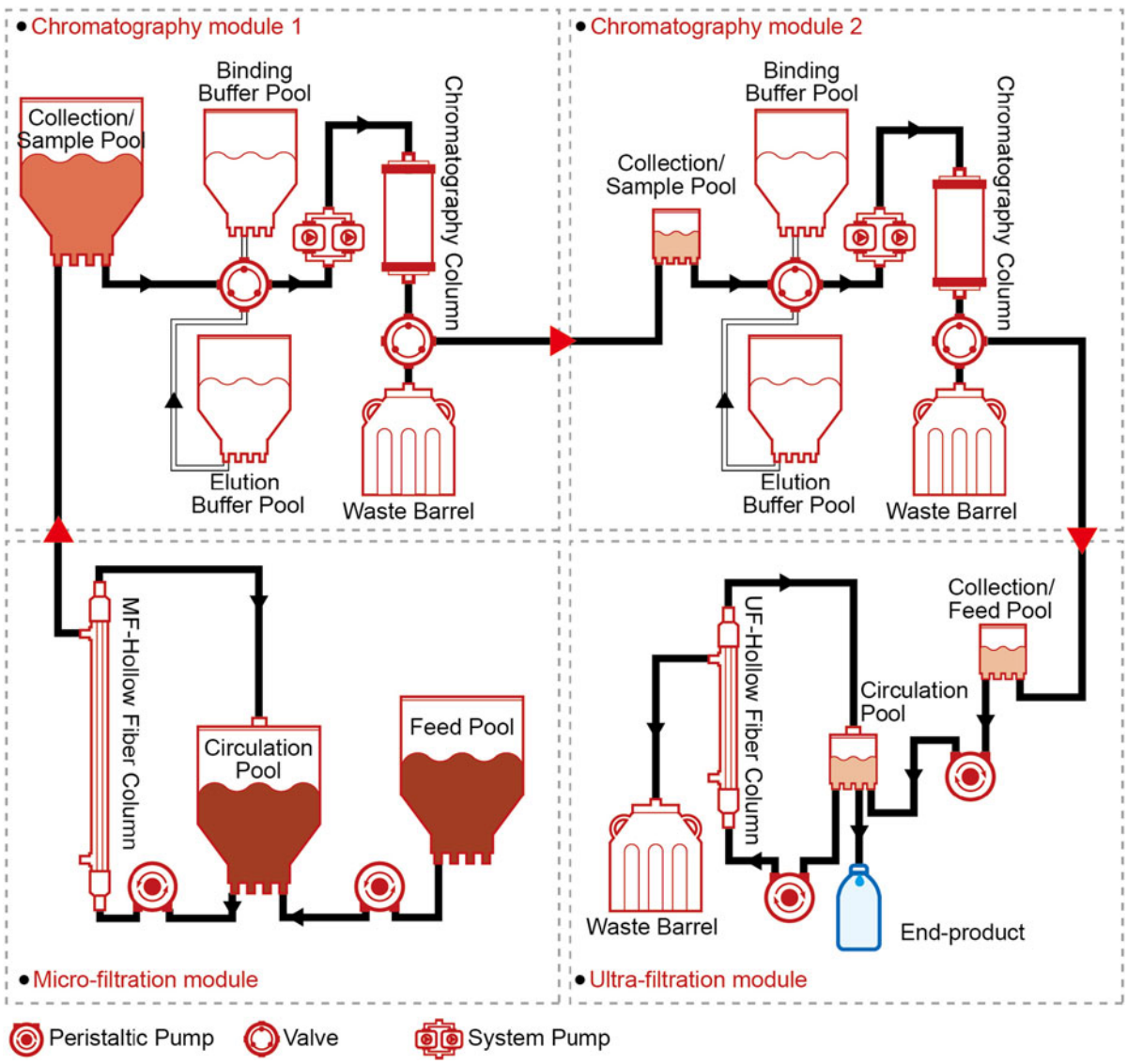

Fig. 1 A versatile design chart of the 'pipeline' system for general mAbs downstream preparation

To construct the chromatography module, an AKTA Avant 150 system was employed. As shown in Fig. 1, storage bags containing buffer or sample, a storage bag used as collection pool, and a barrel used as a waste reservoir were connected via silica-gel pipes and connectors to the inlet valve or outlet valve of the chromatography system. System pumps were installed to provide flow power, and the flow paths were controlled by switching the inlet and outlet valves, so as to choose the appropriate buffers, load samples, and to collect the fractions of the target products. For huAA98 mAb purification, two chromatography modules were constructed: an affinity chromatography module containing a 200 ml MabSelect SuRe column, and a size exclusion chromatography module containing an 1,800 ml Superdex-200 column.

To construct the ultra-filtration module, an ultra-filtration hollow fibre column, a storage bag serving as a circulation pool, a storage bag serving as a feed pool, a sterilized bottle to harvest the end-product, a barrel used as waste reservoir, and 2 peristaltic pumps were connected by silica-gel pipes as shown in Fig. 1.

Upon completion of constructing the individual modules, we utilized silica-gel pipes to connect these modules, in a manner of head-to-tail convergence (Fig. 1). In this study, four modules were employed for the downstream preparation of huAA98.

Pilot scale manufacturing of huAA98 mAb

A batch of 65 l CHO supernatant containing the mAb huAA98 was utilized to test the feasibility of our 'pipeline' preparation processes. After the affinity purification step, a chromatogram was prepared to illustrate the entire capture process (Fig. 2a). Reducing SDS-PAGE (Fig. 2b) indicated that almost all of the fully assembled huAA98 was captured. The SEC-HPLC indicated that in the eluate, the purity of huAA98 was 75 % (Fig. 2c).

During the gel-filtration process, two peak fractions were isolated (Fig. 3a). Reducing SDS-PAGE indicated that the second fraction represented the monomeric form of huAA98 (Fig. 3b), whereas SEC-HPLC established that the purity of the huAA98 in the second fraction was 97 % (Fig. 3c).

At the end of ultra-filtration step, 110 ml of end-product (209 mg total protein) had been collected. Reducing/non-reducing SDS-PAGE (Fig. 4a) and SEC-HPLC (Fig. 4b) indicated that the purity of the huAA98 end-product was 99 %, which was exceeding the purity criteria set by the "Chinese Pharmacopoeia" (Edition 2010).

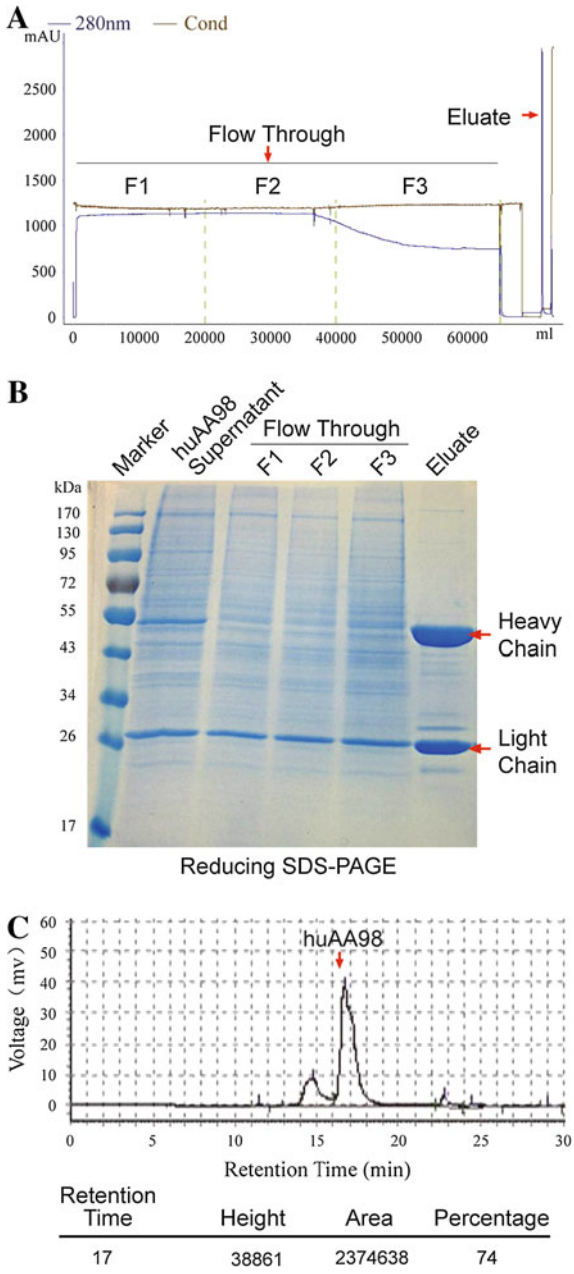

Fig. 2 Results for huAA98 purification by affinity chromatography (MabSelect SuRe media). **a** Chromatogram of the binding-elution step. **b** Reducing SDS-PAGE analysis of fractions isolated by affinity chromatography. **c** SEC-HPLC analysis of the eluate following MabSelect SuRe column purification, with data indicating the purity of huAA98 after the initial step of purification

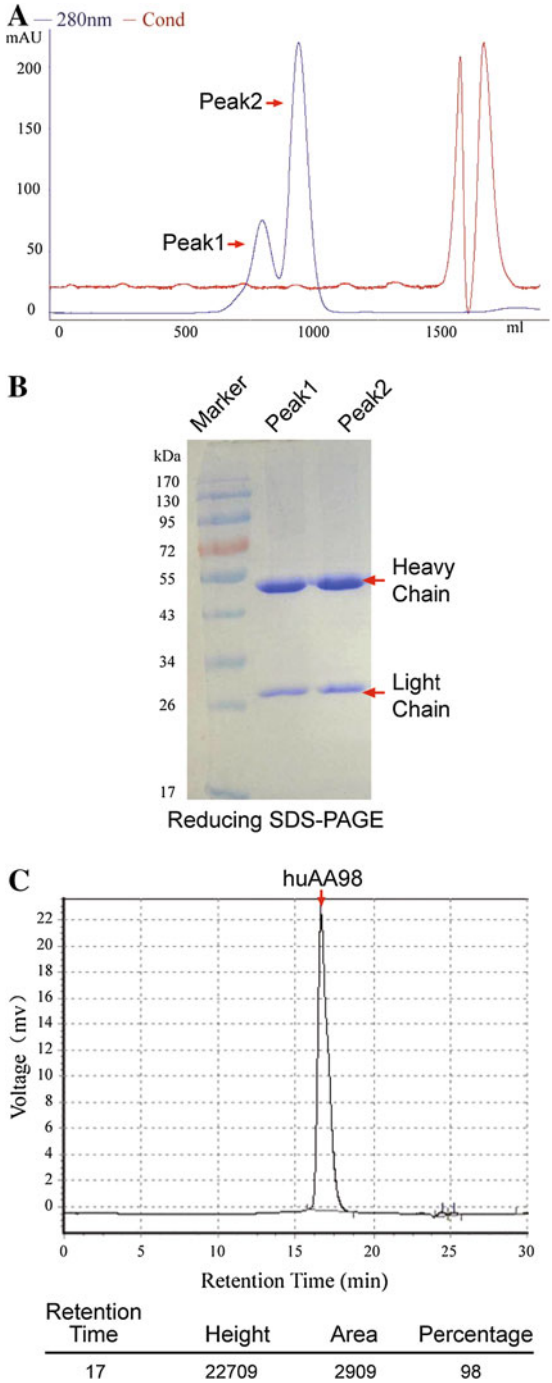

Fig. 3 Results for huAA98 purification by gel-filtration chromatography (Superdex-200). **a** Chromatogram of the gel-filtration step. **b** Reducing SDS-PAGE analysis of fractions isolated by gel-filtration chromatography, *peak 1* and *peak 2* representing purified huAA98 dimer and monomer, respectively. **c** SEC-HPLC analysis of huAA98 monomer, with data indicating the purity of huAA98 monomer after two-step purification

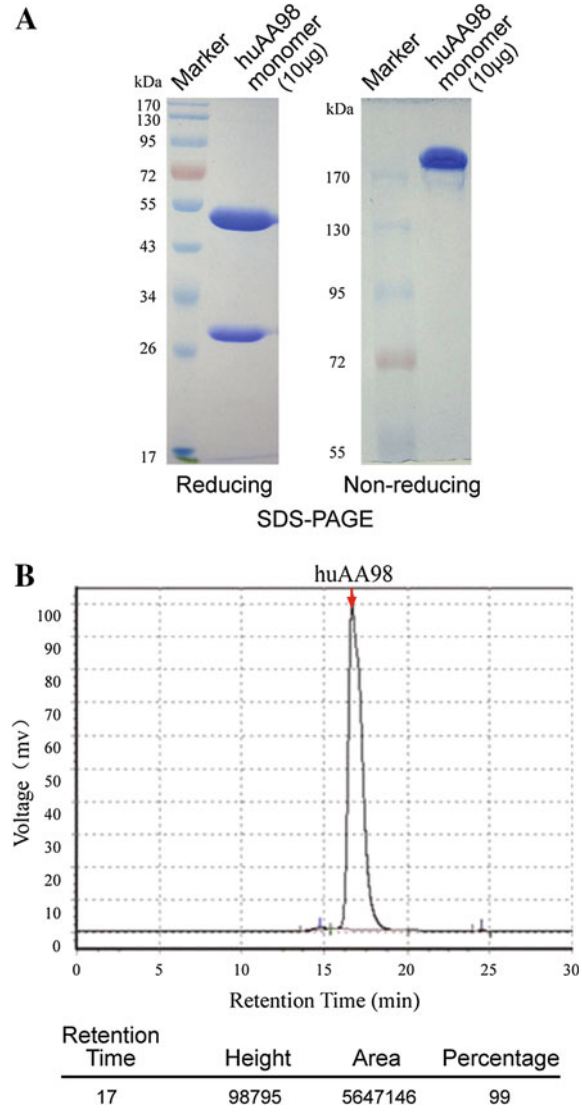

Fig. 4 Purification results of the huAA98 end-product. **a** Reducing and non-reducing SDS-PAGE analysis of the huAA98 end-product. **b** SEC-HPLC analysis of the huAA98 end-product, with data indicating the purity of the huAA98 end-product

Quality control of huAA98 mAb

Endotoxin levels of samples decreased gradually at each step of preparation procedure (Table 1), which is in accordance with the purification progress, with final levels meeting the requirements as set by the Chinese Pharmacopoeia (Edn 2010) (endotoxin <0.3 EU/ml). In addition, the HCP and protein A residue levels (Table 2) were both in compliance with the criteria of

Table 1 Loss of endotoxin during preparation of huAA98

Purification step	Endotoxin (Unit/ml)
1. Culture supernatant	5.16
2. Clarified supernatant after micro-filtration	4.7
3. Eluate from affinity chromatography	1.08
4. Final preparation after gel-filtration chromatography	0.006
End-product: huAA98 mAb preparation after ultra-filtration	0.016

Endotoxin was measured at the end of each step of the huAA98 mAb preparation using a chromogenic substrate method (see Materials and methods)

the Chinese Pharmacopoeia (Edn 2010) (HCP <10 μg/ml, protein A <100 μg/ml).

Biological activity of the huAA98 end-product

Western blot results verified that the huAA98 mAb still recognized CD146 expressed in cell lines (Fig. 5a). In addition, whole cell ELISA verified the binding activity of huAA98 to CD146-positive HUVEC cells; however, no binding was observed for any of the cell lines tested that are lacking CD146 expression (Fig. 5b). These data verified that the end-product huAA98 maintains binding activity to CD146, both at the molecular and the cellular level.

In a tube formation assay, after addition of huAA98, tube formation of HUVECs was suppressed (Fig. 6a). In contrast, human IgG had no such effect. In a migration assay, the motility of HUVECs was also weakened by huAA98, not however by human IgG (Fig. 6b). In summary, our data clearly indicate that the end-product of our preparation procedure has maintained inhibition activity of angiogenesis and migration throughout the entire preparation process.

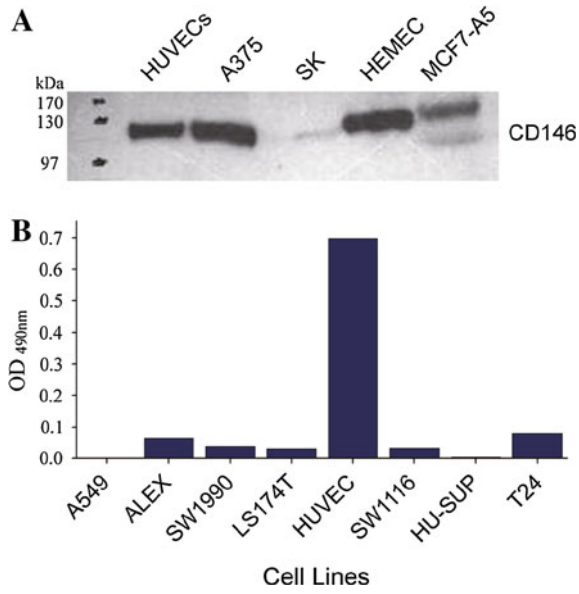

Fig. 5 Results of western blot and whole cell ELISA to determine the binding activity of huAA98 to CD146, two different groups of cell lines were used for analysis by. One group contained cell lines that highly express CD146, including HUVECs, A375, SK, HEMEC and MCF7-A5. The second group of cell lines was chosen for their lack of CD146 expression, namely A549, ALEX, SW1990, LS174T, SW1116, HU-SUP and T24. **a** Western blot analysis of huAA98 mAb binding activity to CD146 expressed in cell lines HUVECs, A375, SK, HEMEC and MCF7-A5. **b** Whole cell ELISA analysis of huAA98 binding activity to surface CD146 expressed in cell lines A549, ALEX, SW1990, LS-1747, HUVECs, SW1116, HU-SUP and T24

Table 2 Overview of the quality assessment for mAb huAA98 end-product

	Concentration (mg/ml)	Purity	Endotoxin (endotoxin unit/ml)	Protein A (ng/ml)	HCP (ng/ml)
huAA98 end-product	6.8	99 %	0.016	1.08	Undetected

Upon completion of the downstream processing, the mAb huAA98 end-product was subjected to a panel of quality tests. Protein concentration, protein purity, endotoxin levels, protein A residues and the host cell protein concentration in the final huAA98 preparation were determined as described in the Materials and methods section, and were all found to be in compliance with the criteria of the "Chinese pharmacopoeia" (Edition 2010)

HCP host cell protein

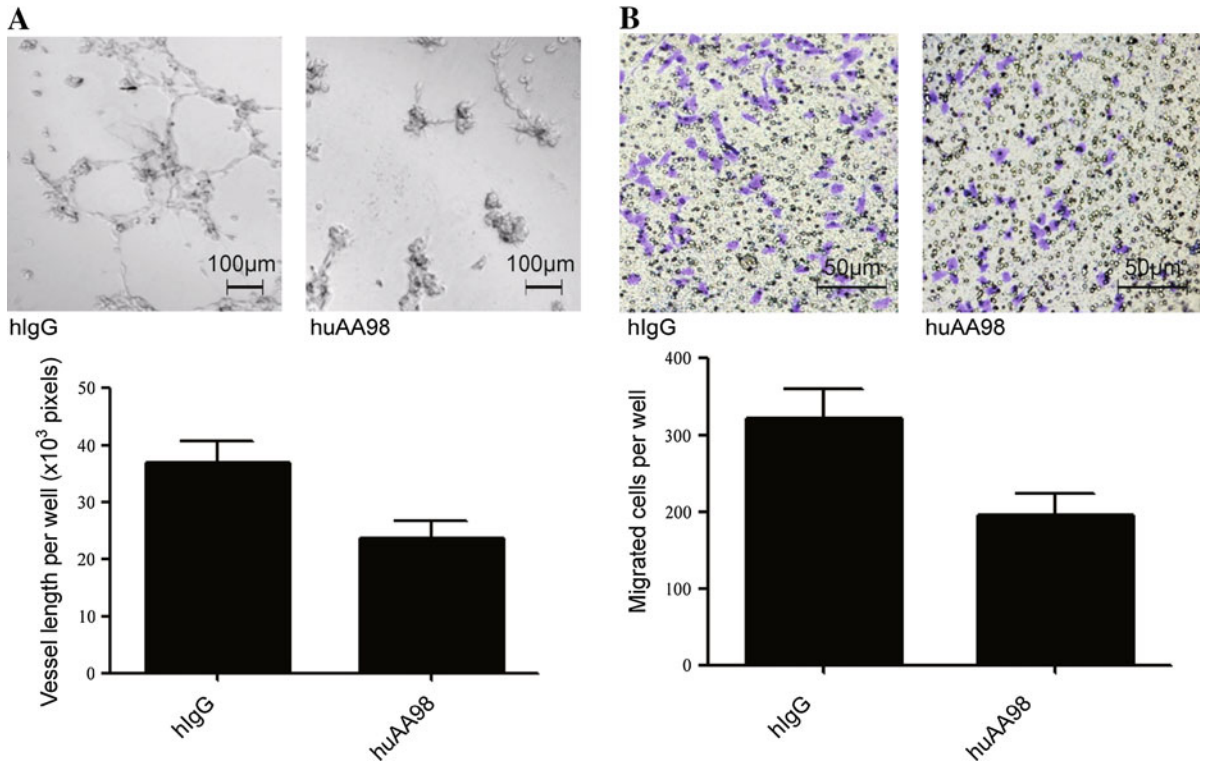

Fig. 6 Inhibitory activity of the huAA98 end-product as measured in tube formation and cell migration assays, with human IgG serving as negative control. **a** Status of tube formation for cell line HUVECs, with or without addition of huAA98, and tube formation calculated as total tube length. *Scale bar* = 100 μm. **b** Status of cell migration for cell line HUVECs, with or without huAA98 treatment; and migration analyzed by counting cells migrating through the filter. *Scale bar* = 50 μm

Advantages of our newly developed 'pipeline' protein preparation system

The preparation system we propose here possesses the following advantages:

(1) The sterile connections between as well as within the individual modules enables the researcher to construct a fully-enclosed environment, which prevents all exogenous contaminations and thus can serve as a mini-GMP facility.
(2) The modular design makes our system sufficiently flexible, so to suit different protein manufacturing processes, and only requires the selection of the appropriate modules, as well as their assembly in a sensible preparation sequence.
(3) Our system is cost-efficient, and can be set up with minimal footprint requirements. When compared to bulky GMP-compliant facilities normally required for manufacturing clinical-grade protein, we have achieved a comparable outcome in terms of product quality, by constructing a miniaturized, sterile preparation environment.

Conclusion

We report for the first time a 'pipeline' system for the downstream preparation of a therapeutic mAb within an environment that is not GMP-compliant, which however, is conforming to requirements for pre-clinical studies. Upon designing such system, a pilot scale downstream preparation of a biologically active therapeutic mAb huAA98 was successfully completed. Furthermore, as a universal platform technology, our system has the potential to solve the problem of large-scale manufacturing of clinical-grade mAbs for research laboratories and early stage drug development organizations, avoiding the requirement for building expensive, GMP-compliant facilities. The application of such 'pipeline' system could facilitate the advance of

the idea of translational medicine, as well as narrow the gap between basic research and clinical application.

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