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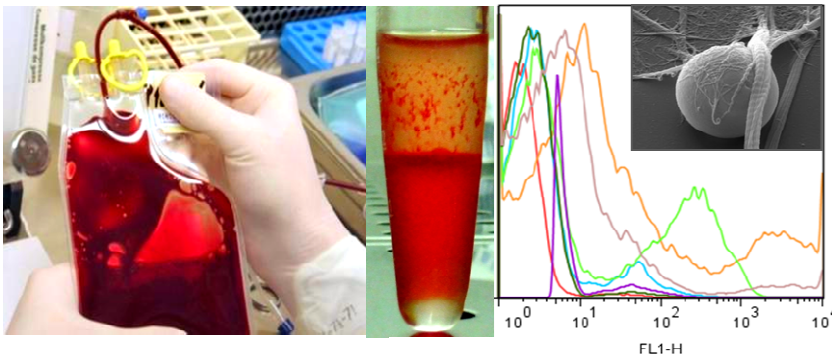


# Optimization of Aqueous Two - Phase System for human Hematopoietic Stem Cell Separation

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Dissertação para obtenção de Grau de Mestre em

**Biotecnologia**

**Júri**

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## Palavras – Chave

Sistema de duas fases aquosas

Purificação por Imunoafinidade

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## Abstract

This thesis aims at the optimization of a novel separation method based on affinity aqueous two-phase system (ATPS) to isolate and purify human hematopoietic stem cells (HSC) from the whole umbilical cord blood (UCB).

The preliminary experiments let to the selection of the prospective affinity ATPS possessing appropriate features for the specific HSC separation, namely a two-phase system composed of 5.6% polyethylene glycol (PEG; 8 000 Da) and 7.5% dextran (500 000 Da) supplemented with 0.15 M sodium chloride (NaCl) and the affinity ligand. A monoclonal antibody (mAb) against the CD34 antigen (anti-CD34) (common cell marker for primitive hematopoietic cells) have been used as a ligand to direct the partitioning of target $CD34^+$ cells towards PEG-rich phase. Antibody (Ab) have been used in a free from, or copolymerized with thermosensitive polymer poly(N-isopropylacrylamide) (poly-NIPAM).

The selected affinity ATPS was successfully used for the specific partitioning and recovery of $CD34^+$ stem/progenitor cells from UCB. It was possible to achieve purification factors (PFs) up to 241 with only one step of partitioning experiment, that makes the implemented system an appealing alternative to traditional techniques for UCB processing, like classical ficoll density gradient centrifugation and magnetic sorting.

The obtained data suggest that this novel selection method can be useful for the development of stem cell engineering, providing easy to apply and cost-effective method for purification of target populations. However, in order to verify the results and assess their statistical significance, all the experiments should be repeated.

## Resumo

O presente trabalho teve como objectivo a optimização de um método de separação por afinidade usando sistemas de duas fases aquosas (ATPS) para isolar e purificar as células estaminais hematopoiéticas (HSC) directamente do sangue do cordão umbilical (UCB).

Experiências preliminares efectuadas com um sistema células modelo (KG1a) identificou o sistema de separação composto por 5.6% de polietileno glicol (PEG; 8 000 Da) e 7.5% dextrano (500 000 Da) suplementado com 0.15 M cloreto de sódio (NaCl) altamente promissor na presença de um ligando de afinidade. De facto, este sistema mostrou possuir as características apropriadas para a separação específica das HSC. Um anticorpo monoclonal (mAb) contra o antígeno CD34 (anti-CD34) (marcador de células primitivas hematopoiéticas) foi usado como ligando capaz de direccionar a partição das células $CD34^+$ para a fase rica em PEG. Os anticorpos (Ab) foram usados livres ou copolimerizados com polímeros termossensíveis de poli(N-isopropilacrilamida) (poli-NIPAM).

O ATPS afinidade selecionado foi utilizado com sucesso para o particionamento específico e recuperação das células $CD34^+$ estaminais/ progenitoras do UCB. Foi possível antigir factores de purificação (PFs) até 241 com apenas um passo de partição, o que faz deste sistema uma alternativa viável para as técnicas tradicionais de processamento UCB como: centrifugação em gradiente de densidade Ficoll e separação magnética.

Os dados obtidos sugerem que este método de selecção poderá ser útil para o desenvolvimento da engenharia de células estaminais, proporcionando fácil aplicação e menor custo para a purificação de populações-alvo.

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# List of Abbreviations

| | |
|---|---|
| AC | Affinity Chromatography |
| ATPS | Aqueous Two-Phase System |
| BERG | BioEngineering Research Group |
| BM | Bone Marrow |
| FACS | Fluorescence Activated Cell Sorting |
| FITC | Fluorescein Isothiocynate |
| FS | Forward Scattering |
| GvHD | Graft versus Host Disease |
| HSC | Hematopoietic Stem Cell |
| IF | Isoelectric Focusing |
| IgG | Immunoglobulin G |
| IMDM | Iscove`s Modified Dulbecco`s Medium |
| IST | Instituto Superior Técnico |
| $K_p$ | Partitioning Coefficient |
| mAb | Monoclonal Antibody |
| MACS | Magnetic Activated Cell Sorting |
| MHC | Major Histocompatibility Complex |
| NaCl | Sodium chloride |
| PBS | Phosphate Buffer Saline |
| PEG | Polyethylene Glycol |
| PF | Purification Factor |
| PFA | Paraformaldehyde |
| pI | Isoelectric Point |
| Poly(NIPAM) | poly(N-Isopropylacrylamide) |
| R | Recovery |
| SC | Stem Cell |
| SDS-PAGE | Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis |
| SS | Side Scattering |
| UCB | Umbilical Cord Blood |

# 1. INTRODUCTION

## 1.1. Background

Hematopoietic stem cells (HSC) are defined by their ability to support hematopoiesis (blood cell renewal), including the generation of mature cells belonging to the main hematopoietic lineages: myeloid and lymphoid (Osawa, et al., 1996). They are the most well characterized stem cell type and this makes them an ideal source for regenerative medicine purposes, mainly for transplantation settings. However, the extremely low frequency of HSCs in any tissue and the absence of a specific HSC phenotype have made their purification a highly challenging goal.

ATPS is an efficient, scalable and cost-effective technique for fractionation and isolation of molecules, and thus, may constitute a powerful tool in the stem cell purification field. An affinity ATPS that allows the successful isolation of HSCs for clinical applications may pave a new way for the development of biotechnology and stem cell engineering, avoiding time-consuming and inefficient procedures.

## 1.2. Aim of studies

The present work aims at the optimization of a novel separation method based on ATPS to isolate and purify human HSC from UCB.

In this master thesis, an immunoaffinity polymer-polymer system composed of PEG and dextran will be tested for selective recovery of human HSC from UCB. A monoclonal antibody (mAb) against the CD34 antigen (common cell marker for primitive hematopoietic cells) will be used as a ligand to direct the partitioning of target $CD34^+$ cells towards PEG-rich phase. Antibody (Ab) will be used in a free from, or copolymerized with a thermosensitive polymer, poly(NIPAM).

In order to define optimal conditions for specific separation of $CD34^+$ cells, a model cell line of human acute myeloid leukemia cells (KG-1a) will be examined using various ATPSs differ in polymers composition and properties. Cell morphology of KG-1a cells after partitioning process will be also studied. Finally, the affinity ATPS established for the initial isolation of $CD34^+$ KG-1a from $CD34^-$ L929 cells will be explored for direct isolation and purification of stem cells from UCB.

# 2. LITERATURE REVIEW

## 2.1. Hematopoietic Stem Cells

Nearly a trillion new blood cells are produced every day in an adult human. This highly active process is maintained by the hematopoietic system that provides a constant supply of blood cells of diverse function. The ability to carry on this activity throughout the lifetime of an individual is dependent on HSCs. These rare, specialized cells with self-renewal capacity and the ability to originate all known mature blood cells, through complex proliferation and differentiation pathways, are drawing lots of attention since their identification by Till and McCullough in 1961 (Till, et al., 1961). Significant knowledge has been gained about human hematopoietic progenitors and they have also been characterized (see Figure 1).

HSCs are thought to reside in specialized microenvironments within hematopoietic tissues formed by supporting cells responsible for HSC survival and self-renewal and regulating their migration and differentiation (Wilson, et al., 2006). The majority of these primitive cells are found within the bone marrow (BM) at a very low percentage (typically 0.01 – 0.05%), but they can also be found in the peripheral blood (PB) and umbilical cord blood (UCB).

Although BM has been traditionally used for HSC transplantation, its UCB counterpart has become a commonly accepted source of HSCs for transplantation settings. It was demonstrated that the stem cell compartment in UCB is less mature when compared to its adult BM equivalent (Buchheiser A, 2009). The biological immaturity, associated with an expanded life span and longer telomeres, indicates a higher proliferative potential *in vitro* and *in vivo*. Moreover, UCB presents a promising alternative to BM since its ease of collection and manipulation, tolerance for the human leukocyte antigen (HLA) – mismatches between donor and recipient, and consequently, a lower risk of graft-versus-host disease (GvHD). However, the low cell number available per unit of UCB makes it still unsuitable for transplantation in adults (Kelly, et al., 2009).

Several methods have been implemented to overcome this limitation, such as clinical trials featuring double cord transplantation (Barker, et al., 2003), intramarrow injection (Francesco, et al., 2010) or ex-vivo expansion of HSC (Jaroscak, et al., 2003) (de Lima, et al., 2008) with promising results.

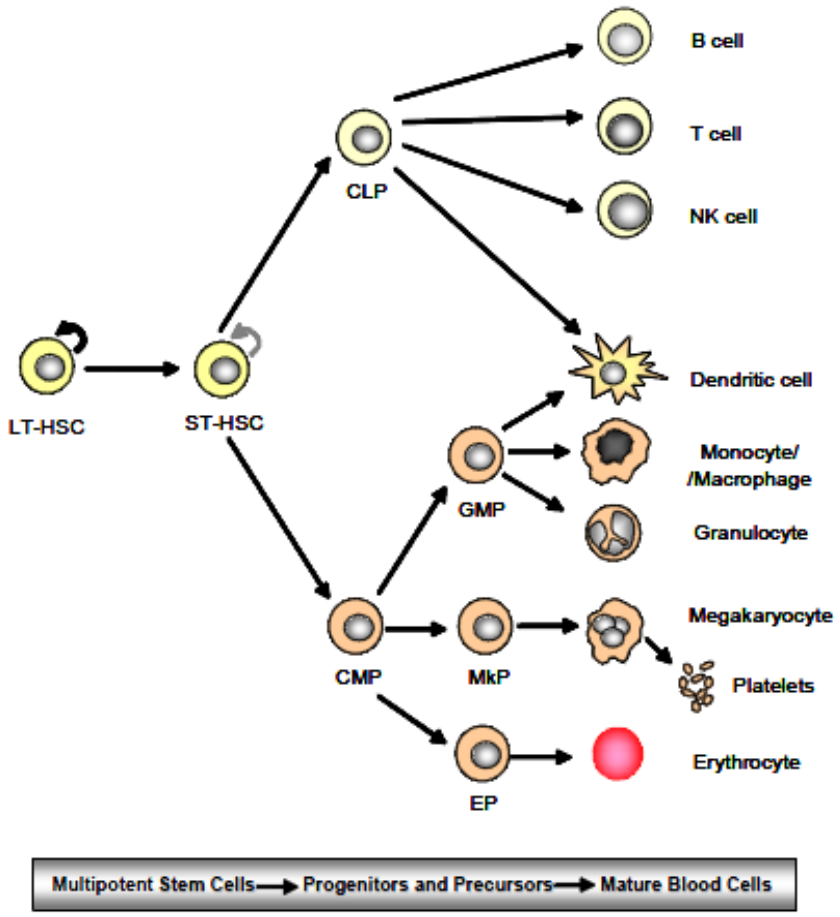


**Figure 1** Schematic representation of the human hematopoietic hierarchy. The circular arrow above LT-HSC and ST-HSC indicates self-renewal capacity. LT-HSC: long-term self renewing HSC; ST-HSC: short-term self-renewing HSC; CMP: common myeloid progenitor; CLP: common lymphoid progenitor; GMP: granulocyte, macrophage progenitor; MkP: megakaryocyte progenitor; EP: erythrocyte progenitor; NK: natural killer. Adapted from (Lobato da Silva, 2006).

### *HSC phenotype and isolation*

Most studies considering HSCs have been directed towards the CD34 expressing cells (Berenson, et al., 1989) (Attar, et al., 2010).

CD34 is a heavily glycosylated type I transmembrane protein that is a member of the sialomucin family of surface molecules (Salati S, 2008). It was reported that developmentally early hematopoietic stem and progenitor cells, small-vessel endothelial cells, and embryonic fibroblasts are expressing CD34. The first CD34 Ab, an anti-My-10 mouse mAb, was raised against the human myeloid cell line KG-1a. The anti-My-10 Ab, an IgG1k against a 115-kd membrane antigen, has been shown to specifically label immature normal human BM cells, including hematopoietic progenitor cells (Civin, et al., 1984). First called the My10 marker, later shown to be CD34. The discovery of CD34 as a hematopoietic cell-surface antigen has revolutionized studies into developmental hematopoiesis and HSC separation techniques. Indeed, it was reported that CD34 cells enriched from marrows of five baboons, using avidin-biotin immunoadsorption, reconstitute the hematopoiesis after high dose of irradiation and this successful engraftment in baboons using $CD34^+$ cells led to their widespread use in human transplantation. Consequently, hematopoiesis rescued in humans after myeloblation have been also noted. Autologous $CD34^+$ cells isolated by immunomagnetic selection (Isolex) reconstituted the hematopoiesis after chemotherapy (Civin, et al., 1996). In another study, allogeneic transplantation of $CD34^+$, that were selected by immunoadsorption (CellPro), gave stable donor hematopoiesis in all patients (Link H, 1996). Therefore, cells enriched for CD34 are routinely used in autologous and allogeneic transplantation, for gene therapy, cell expansion or purging protocols (Nakauchi, 1998) (Engelhardt, et al., 1997).

CD34 Abs are commonly used in clinical settings for the purification of human HSCs and all the current anti-human CD34 mAbs are murine. The Ab against CD34 surface antigen belongs to IgG1 subclass (GE Healthcare, 2002) and the schematic diagram of IgG1 molecular structure is depicted in Figure 2.

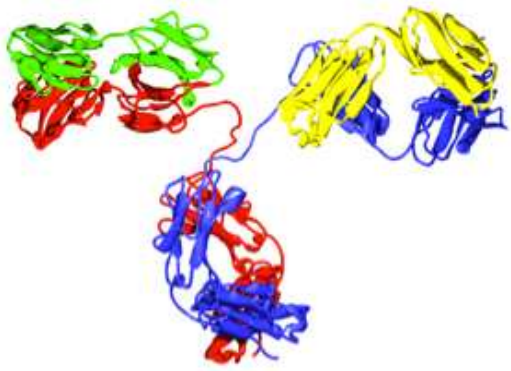

**Figure 2** Schematic diagram of an IgG1 molecular structure: red/blue: heavy chain domain; green/yellow: light chain domain. Adapted from (http://web.mit.edu/vkayser/www/Site/Research.html).

Several studies, however, are providing information that HSC in their quiescent state may not express CD34 surface antigen (Osawa, et al., 1996) (Morel, et al., 1996). Serious questions about expression of this common stem/progenitor marker appeared after experiments performed by Goodel et al. Instead of mAbs, the selection was based on the rapid efflux of the fluorescent DNA-binding dye Hoechst 33342. This allowed identification of the cell population termed side-population (SP) cells. SP cells were revealed to be negative for CD34 expression (Goodell, et al., 1997). SP cells from human UCB have been already characterized (Storms, et al., 2000).

However, the SP cells from other human hematopoietic cell sources, such as BM from normal donors and patients, or mobilized PB from donors and patients with hematological malignancies, have not been extensively characterized, what constitutes a significant limitation for this method.

Methods typically used for HSC separation are based on reaction with specific Abs followed by two alternative methods of isolation – fluorescent activated cell sorting (FACS) or immunomagnetic cell sorting (MACS). The principles of these methods are depicted and described in Figure 3.

Immunomagnetic positive selection of $CD34^+$ cells is performed using paramagnetic microbeads conjugated to specific mAbs (anti-human CD34). In negative selection of $Lin^-$ cells, the unwanted cells are labeled with Abs against known markers for mature hematopoietic cells (CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and glycophorin A) and retained in the column. Non-labeled cells pass through the column and are collected as the $Lin^-$ cell fraction (Jaatinen, et al., 2007).

Flow cytometric enumeration of $CD34^+$ HSCs is widely used for evaluation stem and progenitor cell content in hematopoietic transplantation (Gajowska, et al., 2006). The following section –, Affinity – based separation techniques in chapter 2.2. Methods in cell separation, is devoted to more specific description of FACS technique.

Stem cells are only present in small amounts in adult tissues and organs, thus effective method for specific separation of desired cells while maintaining their viability and functionality is highly important. The existing techniques, however, still have major constraints when the specific degree of performance on a preparative scale is mandatory. Thus, combining several cell-separation steps which differ in capacity and degree of selectivity seems to be the most effective approach. Some examples of separation techniques used for isolation of $CD34^+$ cells are summarized in Table 1.

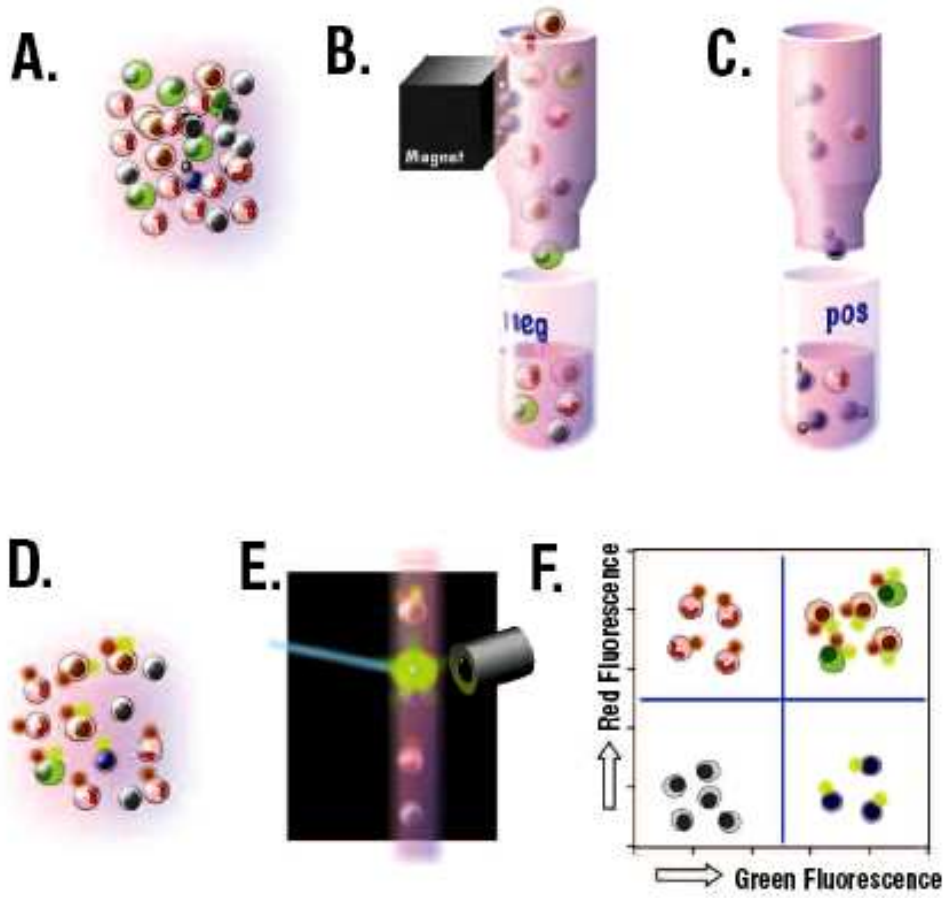


**Figure 3** Enrichment and purification methods for HSCs. Upper panels illustrate column-based magnetic enrichment. **(A)** The cells of the interest are being labeled with magnetic particles that are bound to Abs that only recognize specific cells. **(B)** The cell suspension is passed over a column through a strong magnetic fields, which retains cells with the magnetic particles. **(C)** Other cells flow through and are collected as the depleted negative fraction. The magnet is removed, and the retained cells are collected in a separate tube as the positive or enriched fraction. Lower panel illustrate FACS. **(D)** The cell mixture is labeled with fluorescent markers that emit light of different colors after being activated by light from a laser. Each of these fluorescent markers is attached to a different mAb that recognizes specific sets of cells. The cells are then passed one by one in a very tight stream through a laser beam (blue in the figure) in front of detectors that determine which colors fluoresce is response to the laser. The actual sorting happens by breaking the stream shown in **(E)** into tiny droplets, each containing 1 cell,- that then can be sorted using electric charges to move the drops. Modern FACS machines use three different lasers (that can activate different set of fluorochromes), to distinguish up to 8 to 12 different fluorescence colors and **(F)** sort 4 separate populations, all simultaneously. Adapted from (Domen,J; Wagner, A; Wiessman, IL, 2006).

**Table 1** Some examples of separation techniques used for isolation of CD34+ cells. Adapted from (Dainiak, et al., 2007).

| Technology | Product and company | Cell source | Yield (%) | Purity (%) |
|---|---|---|---|---|
| Immunoselection by magnetic beads | Dynabeads, Invitrogen, Dynal AS | BM | >5 | >30 |
| | | PB | | |
| | | CB | | |
| | Isolex, Nexell | | | |
| | | BM | 40 | 93 |
| | Isolex50, Baxter Healthcare | PB | 53 | 90 |
| | | BM | 70 | - |
| | | PB | 44 | 92 |
| | | CB | 76 | - |
| Immunoselection by submicroscopic colloid magnetic beads | MACS, Miltenyi Biotec | PB | 71 | 97 |
| | | | 77 | 98 |
| | | | 56 | 97 |
| High-speed fluorescence-activated cell sorting | High-speed cell sorter, SyStemix | PB | 60 | 88 |
| Immunoasdorption columns | Ceprate SC, CellPro | PB | 50 | - |
| | | | 53 | 62 |
| | | | 35 | 72 |
| Antibody panning selection | CD34 Collector flask, AIS | BM | 74 | 60 |
| | | | 15 | 33 |

### *Clinical applications of HSC*

UCB HSC transplantation plays an important role in variety of malignant and non-malignant disorders such as leukemia, lymphoma and bone marrow failures. As it was said before, USB constitutes a promising source of HSCs for transplantation settings due to its ease of harvest, availability, less rigorous HLA matching criteria and lower GvHD (Cohen, et al., 2004). However, despite the advantages, the number of UCB HCS transplantations in Europe is still relatively low and data revealed in a 2008 survey showed that they constituted only 7% of the total allogeneic transplantations. Moreover, it was also reported that there were no autologous UCB HSC transplantations in 2008 (Gratwohl, et al., 2010). The low

number of cells collected per unit of UCB limits its use to lightweight recipients, mostly children.

Nevertheless, the significant revolution of UCB HST transplants has been observed and worldwide data provide information about considerable increase in the number of transplants since the first BM transplant performed in 1988 (National Marrow Donor Program, 2011). According to the National Marrow Donor Program this trend will continue to increase exponentially (see Figure 4).

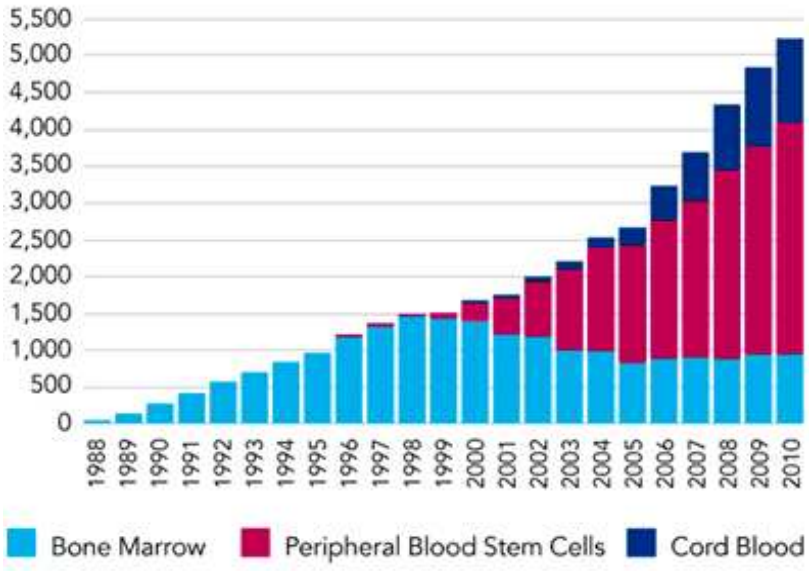


**Figure 4** The utilization of three sources of stem cells; bone marrow, peripheral blood and cord blood. Source: National Marrow Donor Program, 2011.

## 2.2. Methods in cell separation

In general, cells of interests are present only in small amounts together with other major subsets, like fetal cells in maternal blood (Bianchi, et al., 1990), residual leukemic cells from parents (Szczepanski, et al., 2001) or stem cells in hematological samples (Storms, et al., 1999). Dependently on the unique requirements, such as starting sample and downstream application, different methods of isolation can be applied.

Cell separation techniques can be categorized in two distinctive groups. The first approach is based on physicochemical properties, such as size (centrifugal elutriation and density gradient centrifugation), density (density gradient centrifugation), light scattering properties (cell sorter), membrane potential, pH, charge (ATPS and free flow electrophoresis). The latter includes more specific methods, based on affinity interactions. Herein, we can place several methods and among them: cell isolation on affinity matrices, flow cytometry, magnetic bead separation or affinity ATPSs (Dainiak, et al., 2007). The following section, Affinity – based separation techniques, will be entirely devoted to more specific characterization of selection methods based on affinity interactions.

### *Affinity - based separation techniques*

Affinity-based separations, which take the advantage of mAbs that bind to cell-specific surface glycoproteins (Cardoso, et al., 1995) (Assenmacher, et al., 1995) (Martin, et al., 1998) are placed among the most widely used cell fractionation techniques providing desired selectivity.

Flow cytometry provides information about optical and fluorescence properties for a single cell (or any other particle, including nuclei, microorganisms, chromosome preparations, and latex beads). It is a well known method for accurate cell sorting, usually using an automatic device called activated cell sorter (FACS). The simultaneous, parametric model analysis of the physical and/or chemical characteristics is obtained by passing thousands of cells per second through a laser beam and capturing the light of each cell as it passes through. Certain cell population can be resolved by using physical properties, such as size and internal complexity provided by respectively: forward angle light scatter and right-angle scatter (see Figure 5).

Fluorescent dyes can bind or intercalate with different cellular components. Moreover, Abs conjugated to fluorescent dyes can bind specific cell surface proteins. The labeled cells passed by a light source result in excitation of the fluorescent molecules to higher energy state. When returning to their resting states, the fluorochromes emit light

energy at higher wavelengths. Several cell properties can be measured simultaneously by using multiple fluorochromes with similar excitation wevalengths and different emission wavelengths. Commonly used dyes include propidium iodide, phycoerythrin, and fluorescein.

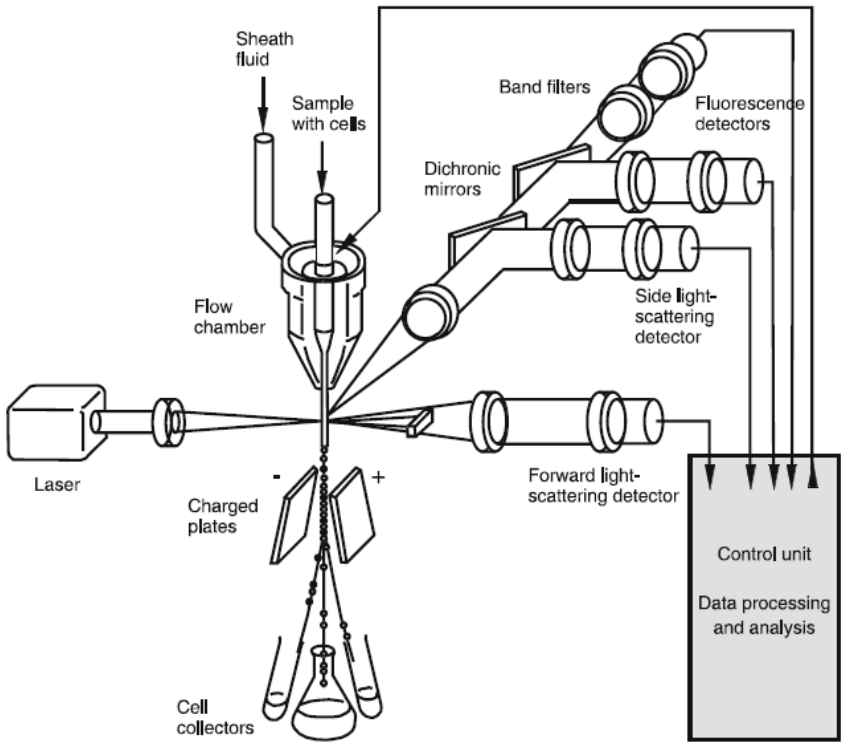


**Figure 5** Schematic drawing of the flow cytometer. Adapted from (Ibrahim, et al., 2007).

Although magnetic separation and flow cytometry are of a great importance due to their accuracy and high selectivity, they are still limited to analytical applications and considered as relatively expensive techniques.

Affinity chromatography (AC) methods are presenting low cost and simplicity (Putnam, et al., 2003). The separation is based on the interaction of cell surface-bound molecules and their complementary ligands (mAbs or lectins). Cell chromatography, however, differs from traditional protein chromatography. Since cells are relatively large, fragile and sensitive to shear stress, the selection process itself poses lots of difficulties. The design of suitable matrix for AC approaches is crucial. Chromatography columns can be

either packed bead or monolithic. Among packed bead chromatography columns we can specify fluidized bead chromatography and conventional adsorbents like agarose (Ghetie, et al., 1978) or silica (Feucht, et al., 1980). The former technique was already used for the separation of monocytes from human PB (Ujam, et al., 2003), however entails the wide range of disadvantages like high shear stress, long equilibration time, narrow range of velocity or recovery of bound cells. The latter procedures are prone to non-specific interactions and fouling. Taking into account time consuming packing process and slow diffusion rates of solutes in packed bead chromatography, the monolithic support seem to be more advantageous. Macroporous, chromatographic matrices with interconnected pores (see Figure 6) of suitable size to enable undisturbed movement of large particles such as mammalian cells, have been reported to be efficient in cell separation (Galaev, et al., 2008).

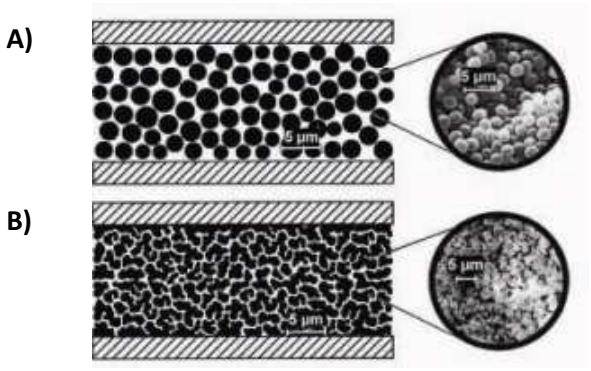


**Figure 6** The examples of chromatographic supports. **(A)** Packed and **(B)** monolithic chromatographic column. Adapted from (Oberacher, et al., 2002).

The selection of mammalian cells using supermacroporous cryogel affinity matrices based on the interaction between cell-surface receptors and an immobilized ligand on a stationary matrix was successfully performed (Kumar, et al., 2010).

To other cell separation methods involving specific interactions we can include affinity ATPSs. The selection is based on specific partition of cells in two immiscible aqueous polymer solutions. The technique allows cell separation on a preparative scale and enables relatively easy collection of the target. Although it was previously reported that in order to

obtain the desired selectivity, the repetitive extractions of objective product are required (Kumar, et al., 2008), a method based on affinity ATPS, that resulted in a high purity population of cells in only one purification step, will be explored in this master thesis. The following chapter, 2.3. Aqueous – two phase systems, is entirely devoted to more specific description of ATPSs.

## 2.3. Aqueous - two phase systems

### *ATPSs – definition*

ATPS, – formed as a result of incompatibility between two aqueous solutions of structurally different components above a certain critical concentration, were firstly described in the literature by Beijernck in the 19$^{th}$ century. He discovered that agar and gelatin form two phases when mixed at certain concentration (Beijerinck, 1896). However, only in the second half of the 19$^{th}$ century the potential use of ATPSs as a powerful separation technique of biomolecules was reported by Albertsson (Albertsson, 1986). ATPSs can be divided into three classes: polymer/polymer, polymer/salt and the thermoseparating systems containing polymer or detergent. The most widely used ATPSs are the PEG/dextran and PEG/salt systems (Cabral, 2007). The selective partitioning of the target product between the two phases is the basis of the two phase system separation. The ATPSs enable processing large quantities of biomolecues at large scale and constitute a gentle separation technique providing a non-toxic and highly hydrated environment (Kaul, 2001).

### *Factors affecting cell partitioning*

Separation in two-phase systems is influenced by several factors, such as: polymer type, molecular weight and concentration, salts type and concentration, pH values and ionic strength (Zaslavsky, 1995). The composition of the systems can be adjusted and modified to properly affect the partition, that is also dependent on target solute properties like molecular weight and surface features, such as charge, lipid composition or presence of specific components. Physical properties of ATPSs, like electrostatic potential difference, interfacial

tension, and phase hydrophobicties have been also reported to play a crucial role in cell partitioning (Albertsson, 1986) (Walter, et al., 1982). The variety of chemical and physical interactions involved in the separation process creates a great number of possible manipulations in the system properties. It is difficult, however, to clarify the actual influence of each of the physical properties by varying the composition of ATPS. Changing one parameter in the composition of the ATPS we can interfere other physical features.

### *Affinity extraction*

Successful applications of ATPS have been implemented for the downstream processing of several biopharmaceuticals (Biopharma: Biopharmaceutical Products in the US and European Markets). This technique allows integration of clarification, concentration and partial purification into one step, however the specificity of the separation is still relatively low. In this respect ATPSs have been modified by introducing affinity ligands, mostly to PEG/dextran systems, to confer proper specificity.

There are two major categories of ligands used in affinity extraction, namely high affinity (polyclonal and monoclonal Abs) and general affinity ligands, such as fatty acids, peptides or triazine dyes (Cabral, 2007). In the latter case, a wide spectrum of interactions with the target product narrow their application and they are rather used in a limited manner.

Antigens or Abs used as affinity ligands have been already applied in separation processes based on immunoaffinity partitioning, in order to separate cells on the basis of their surface receptors (Kumar, et al., 2001).

Polymers which can be soluble/insoluble or/and stimuli responsive have also been implemented as functional ligand carriers for the specific separation of several biological substances (Hoffman, et al., 2007). In such systems, polymers modified with affinity ligands are preferentially partitioned to the top phase and by changing systems conditions, such as temperature or pH, can be precipitated and recovered after partition process. Intelligent polymers with attached Abs (Eudragit S 100 conjugated with IgG) have been already used for

the separation of mouse hybridoma 16-3F cells from mouse myeloma NS-1 cells on the basis of anti-α -amylase Ab production by the former one (Hamamoto, et al., 1996). A temperature sensitive polymer, namely poly(NIPAM), was applied as a ligand carrier for the specific separation of animal cells (Kumar, et al., 2001). The following chapter, 2.4. Applications of thermally responsive intelligent polymer - poly(NIPAM), will be entirely devoted to more specific description of intelligent polymers.

## 2.4. Applications of thermally responsive intelligent polymer – poly(NIPAM)

Poly(NIPAM) (Figure 7) is a temperature-responsive polymer that undergoes a reversible phase transition from a swollen hydrated to a compact dehydrated state at a lower critical solution temperature (LCST) of 32°C. Poly(NIPAM) solutions are thus monophasic below the LCST and biphasic above it. Temperature-responsive intelligent surfaces prepared by the modification of an interface with poly(NIPAM) and its derivatives, have been widely used for biomedical applications (Gil, et al., 2004).

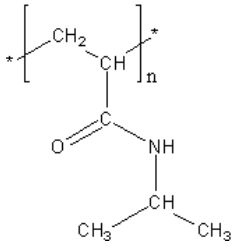


**Figure 7** Chemical structure of poly(NIPAM)( (Gil, et al., 2004).

Hydrophilic/hydrophobic temperature responsive changes followed by temperature alterations give rise to thermally modulated interactions with biomolecules and cells. For instance, temperature-responsive surfaces using the thermosensitive polymer poly(NIPAM) and its derivatives have been already implemented in controlled drug delivery systems (Cammas, et al., 1997) (Chung, et al., 1999) (Kurisawa, et al., 2000), enzyme bioconjugates (Matsukata, et al., 1994) and microfluidics (Yu, et al., 2003). Moreover, poly(NIPAM) grafted surfaces, which exhibit a reversible temperature-dependent phase transition in aqueous at about 32°C (Heskins, et al., 1968) are being used as a new form of

chromatographic supports (Ayano, et al., 2006) (see Figure 8) and cell cultures for tissue engineering (Yamato, et al., 2007).

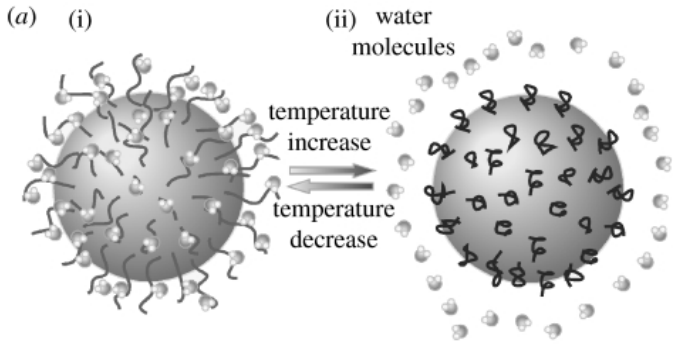


**Figure 8** Concept of temperature-responsive chromatography: **(*a*)** hydration and dehydration of grafted polymer under an external temperature change, **(i)** hydrated, expanded poly(NIPAM) and **(ii)** dehydrated, shrunken poly(NIPAM). Adapted from (Nagase, et al., 2009).

Furthermore, temperature-responsive alterations of poly(NIPAM) have been reported to be successful in thermally modulated attachment and detachment of cells, what can constitutes an alternative method for cells recovery. Such systems are being advantageous since it is possible to detach cells as single and/or confluential cell sheets. In the case of tissue culture on commonly used polystyrene surfaces, harvesting the cells by enzymatic treatment may cause cell-cell connections damage (see Figure 9) (Kumashira, et al., 2010).

Poly(NIPAM) conjugated with Abs have been also reported to be effective in type-specific separation of animal cells using ATPSs. Poly(NIPAM) was used as a ligand carrier for the specific separation of mammalian cells (see Affinity Extraction section in 2.3. Aqueous Two-Phase System chapter) (Kumar, et al., 2001).

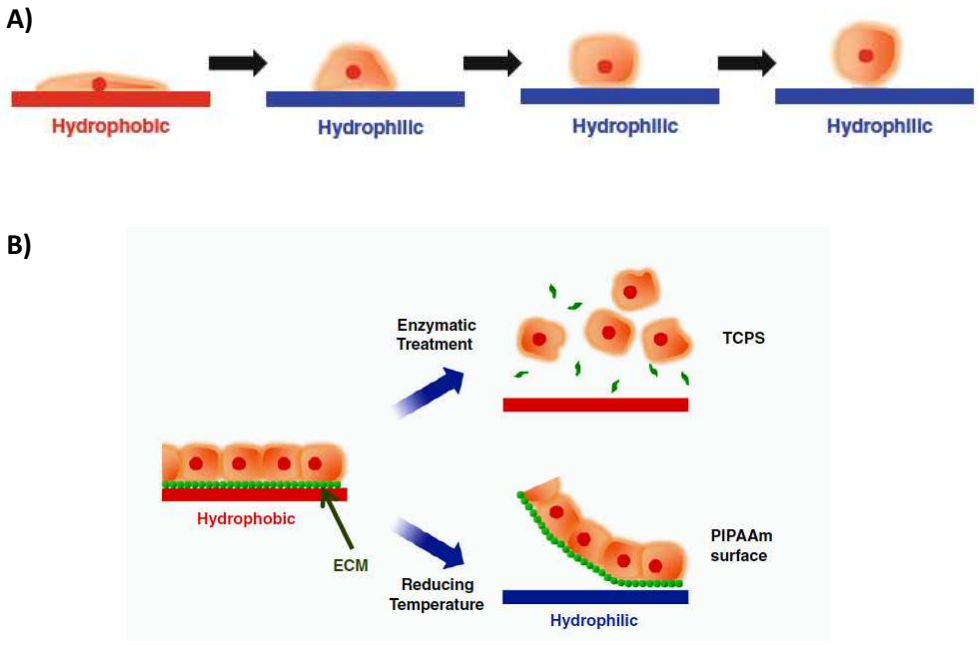


**Figure 9** (**A)** Schematic representation showing the mechanism of cell sheet detachment from poly(NIPAM) surfaces. **(B)** Cell sheet harvested from poly(NIPAM)-grafted surfaces. Cells can attach and proliferate onto temperature-responsive surfaces at 37ºC, whereas lowering temperature to 20ºC facilitates the detachment of cell layers as a cell sheets. Adapted from (Kumashira, et al., 2010).

These novel intelligent surfaces seem to be of significant importance to various biomedical fields and constitute a promising tool in the cell separation techniques.

# 3. MATERIALS AND METHODS

## 3.1. Materials

### 3.1.1. Chemicals

Polyethylene glycol (PEG) with molecular weight (MW) of 8 000 Da was obtained from Sigma (St. Louis, MO, USA), and PEGs with MW of 3 000, 6 000 and 10 000 Da from Fluka (Buchs, Swirzerland). Dextran with MW of 500 000 Da was purchased from Amersham Biosciences (Uppsala, Sweden). NaCl was obtained from Panreac Quimica Sau (Barcelona, Spain). Water used in all experiments was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade.

### 3.1.2. Biologicals

#### *Cell lines – an experimental model*

Human Caucasian acute myelogenous leukaemia cells (KG-1a) derived from bone marrow expressing CD34 surface antigen ($CD34^+$) have been used as a positive control and murine aneuploid fibrosarcoma cells (L929) as a negative control ($CD34^-$).

#### *KG-1a cell cultivation*

The human $CD34^+$ hematopoietic progenitor line KG-1a was cultured in Iscove`s Modified Dulbecco`s Medium (IMDM, Gibco Laboratories) with 10% heat-inactivated Fetal Bovine Serum (FBS) at 37°C in a humidified atmosphere containing 5% $CO_2$. Medium was changed 2-3 times a week. Cell suspension was removed from the flasks and centrifuged at 280*xg* for 7min. The supernatant was discarded and the pellet was ressuspended in growth medium. Trypan blue dye exclusion method (Strober, 1997) was used to determine the number of viable and dead cells in hamacytometer under an optical microscope (Olympus CK40).

### *L929 cell cultivation*

Cells were cultured for 3-4 passages (in T-75 and T-125 flasks with working volumes of 10 ml and 25 ml, respectively) and allowed to grow until confluence IMDM containing 10% Fetal Bovine Serum (FBS), at 37ºC and 5% $CO_2$ in a humidified atmosphere. Medium was changed 2-3 times a week. Near cell confluency (80-90%) exhausted medium was removed from the flasks and cells were washed with phosphate buffered saline (PBS, GibcoBRL) and accutase was added followed by incubation at 37ºC for 7 min. In the next step pre-warmed (37ºC) IMDM with 10% FBS was added in order to stop accutase action and the cell suspension was centrifuged at 280*xg* for 7 min. The supernatant was discarded and the pellet was ressuspended in growth medium. Trypan blue dye exclusion test was used to determine viable and dead cells in hemacytometer under an optical microscope.

### *Human donor cell preparation*

The UCB samples were gently provided by Serviço de Obstetrícia, Centro Hospital Lisboa Norte, Lisboa, Portugal, and were collected after maternal donor consent. The samples were processed within 24h after collection.

### *Antibodies*

Human IgG for therapeutic administration (product name: Gammanorm) was purchased from Octapharma (Lachen, Switzerland), as a 165 mg/ml solution containing 95% of IgG. The mAb against human CD34 antigen (anti-CD34, IgG1) was purchased from Biolegend, as 0.5 mg/ml solution in phosphate buffer, pH 7.2, containing 0.09% sodium azide.

The mAb against human CD34 antigen (anti-CD34, IgG1) was also produced by murine hybridoma cells AC133.1 from the American Type Culture Collection (Manassas, VA, USA) in our laboratory according to the cell line supplier. Anti-CD34 was then purified from culture supernatants using protein G affinity chromatography on a MabSelect column (GE Healthcare, Uppsala, SE).

## 3.2. Methods

### 3.2.1. Preparation of Ab-poly(NIPAM) conjugates

Based on Kumar *et al*. (2001) the protocol for copolymerization of NIPAM with Ab (IgG and anti-CD34) was implemented and the reaction sequence is depicted in Figure 10.

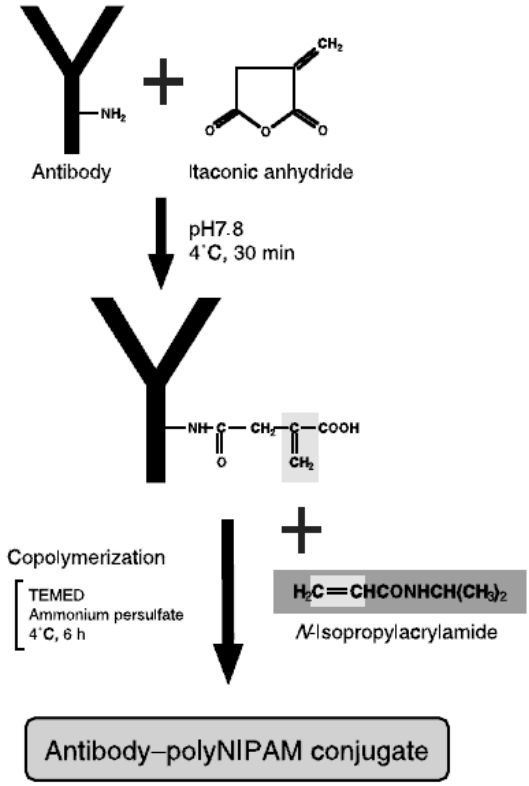


**Figure 10** Modification of Ab with poly(NIPAM). Adapted from (Kumar, et al., 2001).

The solution of mAb against human CD34 antigen (0.4 mL, 0.5 mg protein/mL) purchased from Biolegend was first desalted and concentrated to 15 µl with 0.5 ml Amicon Ultra centrifugal filters from Millipore by following the procedure shown in Figure 11 and in the next step dissolved to 3 ml in 0.2 M PBS, pH 7.8, in the presence of glucose to the final concentration of 5%. This gave a final Ab concentration of 0.066 mg/mL. The same procedure was implemented for human IgG purchased from Octapharma, that was used as a negative control ligand. The final IgG concentration amounted to 0.5 mg/mL.

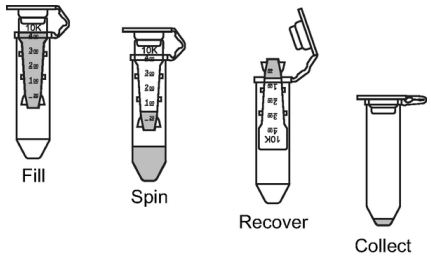


**Figure 11** Sequence of steps leading to desalting and concentration in Amicon Ultra- 0.5mL Centrifugal Filters for Protein Purification and Concentration. Adapted from (www.milipore.com/catalogue/module/c82301, 2011).

Abs dissolved in PBS were then treated with itaconic anhydride (2.75 mg/mL in 0.2 M PBS, pH 7.8, 0.125 mg/mg protein) in order to introduce C-C double bonds into amino groups within an Ab. The reaction mixture was stirred at 4°C for 30min. After incubation, the derivatized Ab solution was dialyzed overnight against ultrapure water at 4°C or desalted in 5 ml HiTrap™ column (GE Healthcare, Uppsala, SE). In case of dialysis, the volume of protein solution after this step became nearly double.

Since it was previously proved (Kumar, et al., 2001) that the maximum copolymerization efficiency was achieved at a high NIPAM content of about 40 mg/mg of protein, 0.05 g (for human IgG) and 0.01 g (for anti-CD34) of NIPAM monomer was added to perform radical copolymerization. The mixtures were flushed with nitrogen gas for 5 min. In the next step, 100 µL of ammonium persulfate (10% w/v) and 10 µl of TEMED was added to the solution. The reaction mixtures were then incubated at 4°C for 6 h.

The reaction was also performed at different conditions, varying the temperature of incubation step in the range between 4-25°C.

Homopolymer of poly(NIPAM) was prepared in the same way in ultrapure water instead of adding Ab solution.

After copolymerization the conjugates solutions of both IgG and anti-CD34 had Abs concentration of 0.22 mg/ml and 0.033 mg/ml.

### 3.2.2. Poly(NIPAM) conjugates - analytical determinations

Thermoprecipitation of Ab-poly(NIPAM) conjugates from aqueous solution, monitored as turbidity, was obtained by measuring the optical density at 470 nm at different temperatures with a spectrophotometer equipped with a thermoregulated cuvette.

The concentration of Abs conjugated with poly(NIPAM) was determined by analytical protein G chromatography using a PG ImmunoDetection sensor cartridge from Applied Biosystems (Foster City, CA, USA). The binding buffer was composed by 10 mM phosphate, 150 mM NaCl, pH 8.4 and the elution buffer was composed by 12 mM HCl, 150 mM NaCl, pH 2-3. Samples were previously diluted in binding buffer. The concentration of Abs amounted value of 25.94 μg/mL.

### 3.2.3. Determination of phase diagrams

Binodal curves were determined by the cloud point method for systems without NaCl and by turbidimetric titration method for systems containing NaCl (Hatti-Kaul, 2000). In the former, PEG and dextran solutions were added drop-wise to each other and mixed until the resulting mixture became cloudy, while in the latter, two-phase systems were prepared and a NaCl solution was added drop-wise until the mixture became clear. Graphical representation of the methods is depicted in Figure 12 below.

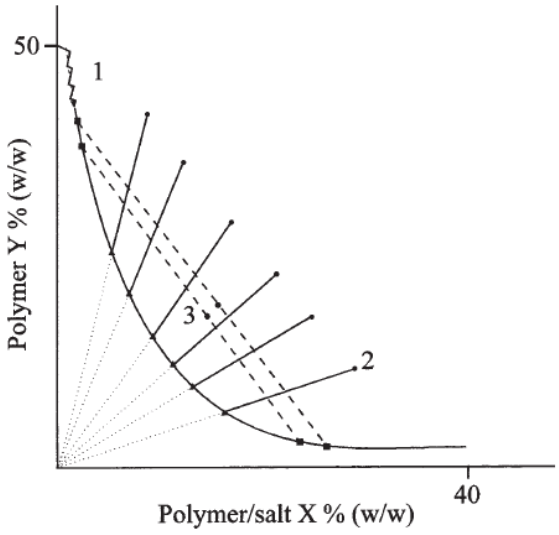


**Figure 12** Graphical representation of three methods used to determine the binodal. 1. Illustrates the cloud point method, where a concentrated stock of component 1, i.e., polymer/salt X (in our case, dextran 500 000) is added to a concentrated stock of component 1; 2, i.e., polymer Y (in our case, PEG). The solution is repeatedly taken above and below the cloud point, by the addition of water, and the process is repeated 2. Illustrates turbidometric titration where a series of systems (●) are prepared and titrated until one-phase system is formed – the binodal lies just above this point (▲). Adapted from (Hatti-Kaul, 2000).

### 3.2.4. Aqueous two-phase systems partitioning

PEG/dextran ATPSs were prepared by weighting the appropriated amounts of components from stock solutions of 50% (w/w) PEG (MW 3 000, 6 000, 8 000 and 10 000 Da), 25% (w/w) dextran (MW 500 000 Da), water and NaCl solution (0 – 0.5 M). All systems were prepared in graduated centrifuge tubes with a total phase system mass of 2.5 g or 5 g. The final composition of the systems was calculated taking into consideration the amounts of affinity ligands and cell extract solutions that were then added to the systems.

The Ab-poly(NIPAM) conjugates (0.1 mL) or solution containing only anti-CD34 "free" Abs were mixed with a 0.1 mL cell suspension and incubated for 30 min under laminar flux chamber at room temperature.

The same procedure was maintained for control experiments, however instead of Ab-conjugates or anti-CD34 "free" Abs, equal amount of water, IgG, IgG-poly(NIPAM) or unconjugated poly(NIPAM) solution was added. All system components were then thoroughly mixed in a vortex (Ika, Staufen, Germany) and phases were left to separate at 25ºC for 60 min in sterile conditions. To ensure complete phase separation, the systems were centrifuged at 180*xg* for 5 min. After centrifugation cells were collected from the interface, appropriately diluted in PBS (10 mM phosphate, pH 7.4, sterile, filtered) and counted using trypan blue dye exclusion method (Strober, 1997). Similarly, some cells were collected from the top and bottom phase after centrifugation. The schematic presentation is depicted in Figure 13.

### *Antibody partitioning studies in both forms, conjugated and free in solution*

The partitioning of Abs (anti-CD34 or IgG), either free in solution or conjugated with poly(NIPAM) was performed in 15 mL graduated centrifuge tubes with a total mass of 2.5 g for each system. Two systems with different compositions were prepared and evaluated: (i) 4% PEG 8 000 and 5% dextran 500 000; (ii) 5.6% PEG 8 000 and 7.5% dextran 500 000. For both systems the effect of NaCl was studied by varying the concentration of salt from 0 to 0.2 M.

After partitioning phase volumes were measured and samples from both top and bottom phase were taken for HPLC analysis. The concentration of Abs was determined by analytical protein G chromatography using a PG ImmunoDetection sensor cartridge from Applied Biosystems (Foster City, CA, USA). The binding buffer was composed by 10 mM phosphate, 150 mM NaCl, pH 8.4 and the elution buffer was composed by 12 mM HCl, 150 mM NaCl, pH 2-3. Samples were previously diluted in binding buffer.

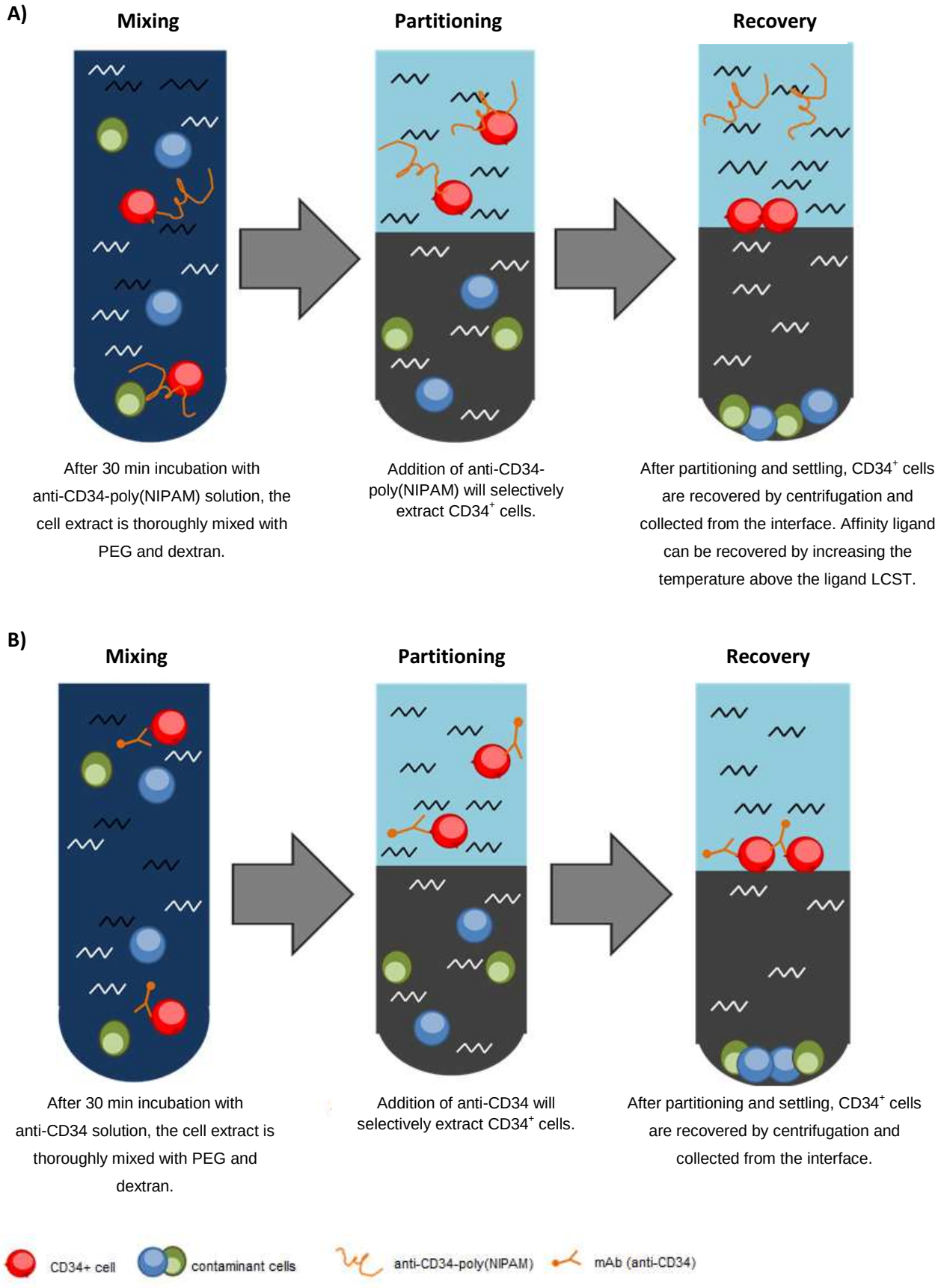


**Figure 13** Schematic overview of specific partitioning of the cells in ATPSs containing affinity ligands: **(A)** anti-CD34-poly(NIPAM) and **(B)** anti-CD34, respectively.

### *KG-1a cell partitioning studies*

Systems with different compositions were screened to determine the best conditions for hHSCs separation, using KG-1a cell line as a model system. The anti-CD34 and IgG conjugates partition studies were performed in 15 ml graduated centrifuge tubes with a total mass of 2.5 g for each system. The same procedure was followed for systems containing only "free" anti-CD34 Abs as affinity ligands. Several systems were prepared with different PEG MW (3 000, 6 000, 8 000, 10 000 Da) to determine the best conditions for specific separation. For the system composed by PEG 8 000 and dextran 500 000, two different overall compositions were evaluated, namely: (i) 4% PEG 8 000 and 5% dextran 500 000; (ii) 5.6% PEG 8 000 and 7.5% dextran 500 000. The effect of NaCl was also studied by varying the concentration of salt from 0 to 0.15 M in case of systems comprising Ab-poly(NIPAM) conjugates, and 0 to 0.5 M in case of systems with only "free" mAb anti-CD34 as affinity ligands.

The cell extract was applied to the ATPS in the same manner as described above (see Figure 13). After 60 min the ATPSs were centrifuged at 180*xg* for 5min and the cells settled on the interface were collected and diluted in PBS. Subsequently cells were counted using trypan blue dye exclusion method. Similar procedure was followed for cells collected from the bottom phase after centrifugation.

### *Cell mixture partitioning studies*

For the model system to separate KG-1a cells ($CD34^+$) from L929 cells ($CD34^-$), the two populations were mixed in different proportions ranging from 20 to 80% for a total number of 1.75 x $10^6$ cells per ATPS tube. The cells were collected from T-flasks by centrifugation at 180*xg* for 5min as described previously (see KG-1a cells cultivation and L929 cells cultivation in 2.1.2. Biologicals section). After centrifugation cells were suspended in PBS and cell mixtures containing different ratios of KG-1a and L929 (20:80, 30:70, 70:30, 80:20) were applied to the ATPS after 30 min incubation in Ab-poly(NIPAM) conjugates or anti-CD34 solution respectively. The ATPSs were left to settle for 60 min at room temperature in sterile conditions.

After centrifugation (180*xg*, 5min) cells were collected from the interface and analyzed by flow cytometry.

### *UCB partitioning studies*

UCB partitioning studies were performed in 15 ml graduated centrifuge tubes with a total mass of 5 g for each system. Approximately 1.2 $\times$ $10^9$ cells were incubated with anti-CD34, IgG, anti-CD34-poly(NIPAM), IgG-poly(NIPAM), unconjugated poly(NIPAM) and water for 30 min at room temperature. Cells previously incubated in sterile conditions with different conjugates/Abs solutions were added to the systems, mixed gently with vortex and left for 60 min to separate.

To ensure complete phase separation, systems were centrifuged at 180*xg* for 5min. After centrifugation cells were collected from the interface, top and bottom phase as described above and the number of cells was accessed using hemacytometers (trypan blue dye exclusion method). Cells were also analyzed by flow cytometry.

The purification factor (PF) was estimated as a ratio of the initial percentage of $CD34^+$ cells in the whole UCB samples and after partitioning in the ATPS:

$$PF = \frac{\text{CD34+ cells collected from the interface (\%)}}{\text{CD34+ cells in initial UCB sample (\%)}}$$

The percentage of $CD34^+$ recovered cells (% R) was calculated by the ratio of the number of $CD34^+$ cells obtained after partitioning in the top phase and the number of $CD34^+$ cells present in the initial UCB sample. The number of $CD34^+$ cells present in a sample can be determine by multiplying the percentage of $CD34^+$ cells by the total number of cells present in the sample.

% R was calculated as follows:

$$\% R = \frac{\#3 \times \#4}{\#1 \times \#2}$$

in which, 1 is the total number of cells that will be separated, 2 is the percentage of desired cells in starting population, 3 is the total number of cells in enriched fraction and 4 is the percentage of desired cells in enriched fraction.

### 3.2.5. KG-1a cell morphology studies after partitioning

After partitioning in ATPSs (7.5% dextran 500 000/ 5.6% PEG 8 000) KG-1a cells were collected from the interface and studied. Different conditions were applied to ATPSs, namely increasing concentration of salt (0 - 0.5 M NaCl in case of mAb used as an affinity ligands and 0 - 0.15 M in case of Ab-poly(NIPAM) conjugates), in order to determine the effect of affinity ligands and salt on the cell morphology. KG-1a cells were collected after partition studies, diluted in PBS, counted using trypan blue dye exclusion method and centrifuged at 280*xg* for 7 min. The supernatant was discarded and the pellet was ressuspended in growth medium. Cells were cultured for 7 days (T-25 and T-75 flasks with working volumes of 5 ml and 15 ml respectively) in IMDM containing 10% FBS, at 37ºC and 5% $CO_2$ in a humidified atmosphere. Light scattering and immunophenotypying by flow cytometry (CD34), light microscopy observations and cell counting using trypan blue dye exclusion method (Strober, 1997) were used to characterize the morphology of cells. KG-1a cells were examined directly after partition process and after 3, 5 and 7 days of cultivation. Flow cytometry was performed directly after the partition process and after 7 days of cultivation.

### 3.2.6. Flow cytometry samples preparation

The cells were centrifuged for 7 minutes at 280*xg*, ressupended in PBS and split onto FACS tubes (100 µL). The goat anti-mouse IgG (H+L) (Alexa Fluor 488-conjugated) Abs for anti-CD34 (Invitrogen) were used to stain samples collected from systems containing "free" anti-CD34 as affinity ligands and mouse anti-human mAbs (FITC conjugated) against CD34, IgG1 (BD Biosciences) were used for other systems. The Ab was added to each FACS tube and incubated for 15 minutes at room temperature in the dark. To remove the excess of Ab (not labeling the cells), PBS was added and the cells were centrifuged for 5 minutes at 180*xg*. Then, the cells were ressupended and fixed in 2% paraformaldehyde (PFA) (Sigma) and stored at 4ºC (only if the analysis was not performed immediately). After staining, the cells were analyzed by flow cytometry (FACSCalibur equipment, Becton Dickinson, San Jose, CA) that quantitatively determine the expression of the surface marker. The results were acquired with the software CellQuest (Becton Dickinson®) and analyzed using flow cytometry analysis software FlowJo.

Isotype controls were also prepared for every trial. For the experiments with indirect staining (the goat anti-mouse IgG (H+L) (Alexa Fluor 488-conjugated) Abs, appropriate controls with the secondary Ab alone were performed. This is necessary to estimate non-specific binding of this secondary Ab to non-target cells and to define the level above which fluorescence intensity can be considered specific.

### 3.2.7. Isoelectric focusing

The determination of proteins isoelectric point was performed by isoelectrofocusing on a PhastSystem electrophoresis apparatus from Pharmacia (Uppsala, Sweden). A horizontal gel with a pH gradient of 3 – 9 from Amersham Biosciences (Uppsala, Sweden) was used. Gels were firstly stained with Coomassie Brilliant Blue and, whenever the intensity of the bands was too low, the gels were subsequently stained with silver nitrate.

### 3.2.8. Cell Imaging

For different experiments, cell culture images were taken using optical microscope Olympus CK40.

# 4. RESULTS AND DISCUSSION

## 4.1. Phase diagrams

A phase diagram is composed of a binodal curve and tie lines. The binodal curve constitutes the border line between two phases and the tie lines give the polymers concentration of the two phases in equilibrium. Phase diagrams for dextran 500 000 and PEGs differ in MW (MW 3 000, 6 000, 8 000 and 10 000 Da) were determined for preliminary partitioning experiments aiming to select the proper composition of ATPS resulting in equal volumes of the phases (see Figure 14).

**Figure 14** Photograph of 4% PEG 8 000/ 5% dextran 500 000 ATPS. It can be observed that volumes of bottom and top phase are equal.

Figure 15 A shows the phase diagrams determined for PEG at different MW, and dextran 500 000. It can be seen that for lower polymer MWs, phase separarion occurs for higher PEG and dextran concentrations. Figure 15 B compares binodal curves of PEG 8 000 and dextran 500 000 in the absence of NaCl, with the binodals obtained in the presence of salt. The concentration of NaCl in ATPSs ranged from 0 - 0.15 M. The increasing ionic strength of the medium lead to slight decrease in the concentration of PEG and dextran needed for the two-phase formation.

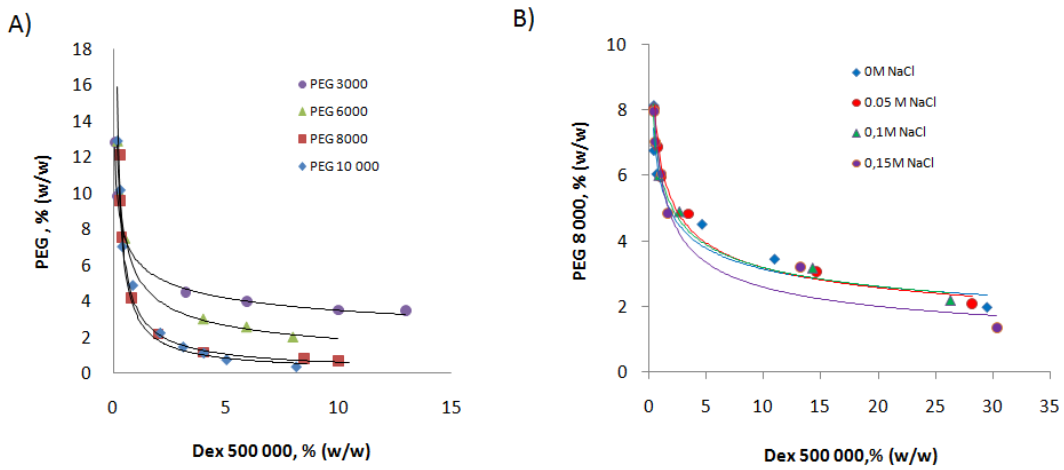


**Figure 15 (A)** Binodal lines for PEG of different MW and dextran 500 000 determined by cloud point method at 23°C. For lower polymer molecular weights, phase separarion occurs for higher PEG and dextran concentrations. **(B)** Binodal lines for PEG and dextran 500 000 system with varying concentration of salt determined by turbidimetric titration.

It should be noted, however, that synthetic polymers are usually polydisperse and their molecular weight distributions may vary from lot to lot even if they are derived from the same manufacturer. Moreover, binodals positions of the phase diagrams for the systems formed by different polymer lots differ accordingly (Zaslavsky, 1995). The position of the binodal for a given polymer system cannot be regarded as a physico-chemical constant of the system.

Since it was previously reported in the literature that the slope of the tie line (STL) value of an ATPS is usually constant within the experimental error limit (Zaslavsky, 1995), i.e. tie lines are parallel to each other, the mean value of the STL for a given system was derived from the literature data and used for composition determination of the ATPSs. The polymer composition of the ATPSs is summarized in Table 2.

**Table 2** Polymer composition of the aqueous dextran 500 000 – PEG (3 000, 6 000, 8 000, 10 000 Da) systems.

| ATPS system | PEG % wt. | dextran % wt. | STL |
|---|---|---|---|
| PEG 3 000/ dextran 500 000 | 6 | 6.5 | -0.435 ± 0.013 |
| PEG 6 000/ dextran 500 000 | 4.5 | 7 | -0.464 ± 0.028 |
| PEG 8 000/ dextran 500 000 | 4 | 5 | -0.486 ± 0.013 |
| PEG 10 000/ dextran 500 000 | 4 | 7.5 | - 0.488 ± 0.029 |

## 4.2. Preparation and characterization of Ab-poly(NIPAM) conjugates

The Ab-poly(NIPAM) conjugates were prepared as depict in Figure 10 (see chapter 3.2. Preparation of Ab-poly(NIPAM) conjugates). The coupling of cyclic itaconic anhydride was used in order to introduce polymerizable vinyl groups to the Ab molecules. Reaction buffer contained 5% of glucose since it was previously reported that presence of glucose during reaction stabilizes the activity of the proteins (Galaev, et al., 1993). Based on previous results (Kumar, et al., 2001), indicating the best optimum range of NIPAM used in the copolymerization reaction, the content of about 40mg/mg-protein was applied to the reaction mixture.

### *Thermoprecipitation studies*

Both Gammanorm IgG and anti-CD34 were modified by poly(NIPAM). Ab-poly(NIPAM) conjugates exhibit a reversible phase transition behavior at 32-35°C. This phenomena is occurring because of the gradual increase in intra and intermolecular hydrophobic interactions with increasing temperature. The homopolymer of poly(NIPAM) was soluble in water below 32°C, and it precipitated sharply as the temperature was raised above this temperature, in other words it demonstrated LCST at around 32°C (Heskins, et al., 1968). Once the Ab molecules were introduced to the carrier backbone, the LCST of the copolymer was higher, reaching values up to 34-38°C (Figure 16). This is because of the protein molecules that render the polymer backbone more hydrophilic.

Different conditions were tested in order to improve the co-polymerization efficiency. One of these was the co-polymerization temperature, which was increased from 4°C to 25°C. It was observed that when the reaction steps were carried out in the 25°C, the Ab-polymer precipitation occurred at higher temperatures, namely 35°C instead of 33°C for the IgG-poly(NIPAM), which indicates that probably more Ab molecules have co-polymerized with NIPAM. However for polymers that should be used in ATPS for separation of biomolecules, it is important that the cloud point is not too high as this can lead to denaturation of Abs that are attached to the polymer backbone. Increased incubation temperature was also not advantageous from engineering process point of view and for

further analysis Ab-poly(NIPAM) conjugates were prepared according to the beginning protocol.

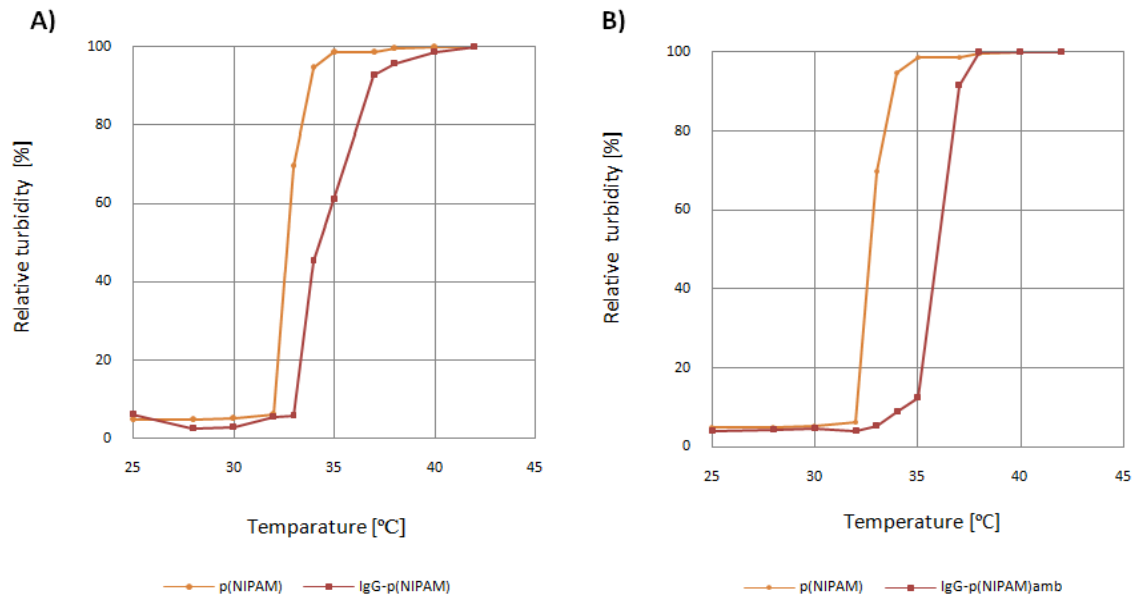


**Figure 16** Thermoprecipitation of Ab-poly(NIPAM) conjugates from aqueous solution (pH 7.4), monitored as turbidity at 470nm. The maximum absorbance obtained was taken as 100% relative turbidity. **(A)** Precipitation of IgG-poly(NIPAM) according to the beginning protocol and **(B)** precipitation of IgG-poly(NIPAM) prepared at 25°C temperature. Conjugates prepared at room temperature demonstrated higher LCST temperature.

As already mentioned, the Ab-poly(NIPAM) conjugates exhibit a higher LCST due to the higher hydrophilicity of these molecules in comparison to the NIPAM homopolymer. One way to reduce the transition temperature is to increase the ionic strength of the medium. This will enhance the precipitation conditions by promoting hydrophobic interactions. When electrolytes are introduced into the solution, the solvation of the polymer by the water molecules has to compete with the solvation of the ions. In the presence of 0.2 M NaCl, the Ab-poly(NIPAM) precipitation occurred almost at the LCST of the NIPAM homopolymer. The same situation was observed for both anti-CD34-poly(NIPAM) and IgG-poly(NIPAM) conjugates. This indicates that thermosensitivity can be tuned to respond to ionic changes of the aqueous media (see Figure 17).

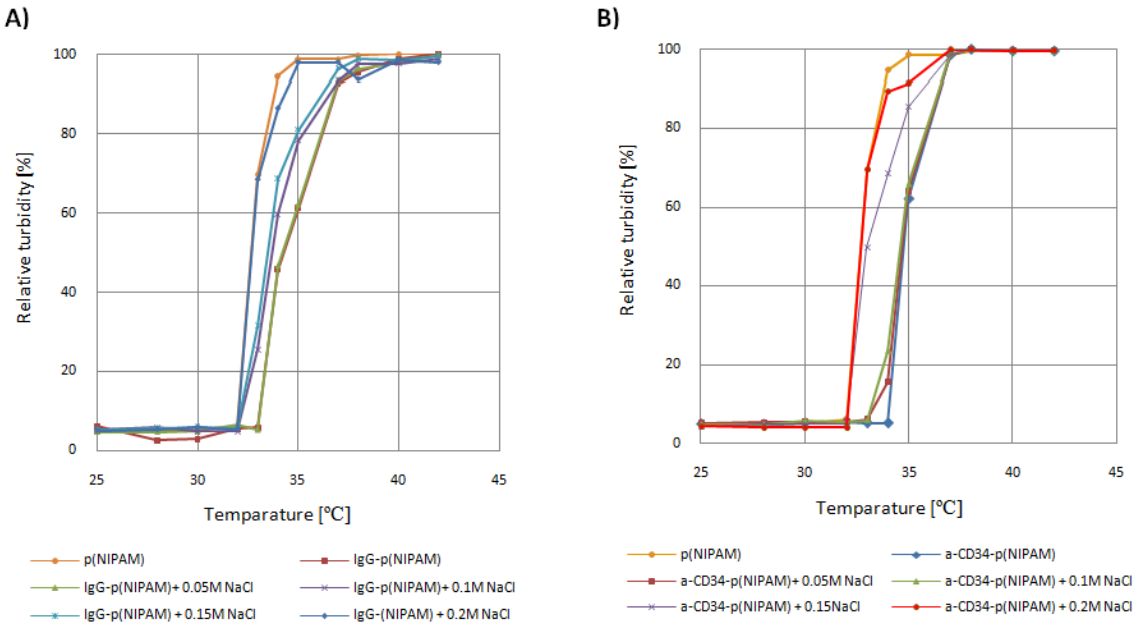


**Figure 17** Thermoprecipitation of Ab-poly(NIPAM) conjugates from aqueous solution monitored as turbidity at 470nm. The maximum absorbance obtained was taken as 100% relative turbidity. **(A)** Precipitation of IgG-poly(NIPAM) and **(B)** anti-CD34-poly(NIPAM) with NaCl concentrations of 0 M, 0.05 M, 0.1 M, 0.15 M and 0.2 M compared to precipitation temperature of poly(NIPAM) homopolymer. It is shown that increasing precipitation conditions are diminishing the cloud-point temperature.

Complete partition of Ab-poly(NIPAM) conjugates to the top phase was observed after heating the ATPS to around 33 or 35°C, depending on the polymer conjugate that was previously added to the system, since only the top phase became milky white, while the bottom phase remained transparent (see Figure 18).

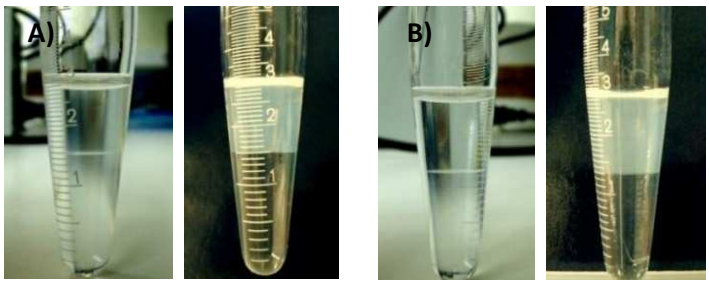


**Figure 18** Photographs of 4% PEG 8 000/ 5% dextran 500 000 ATPS containing Ab-poly(NIPAM) conjugates. Precipitation of **(A)** IgG-poly(NIPAM) and **(B)** anti-CD34-poly(NIPAM) conjugates from aqueous solution (pH 7.4) after heating to 35°C.

### *Ab-poly(NIPAM) partitioning studies*

The partitioning behavior of the Ab-poly(NIPAM) conjugates was studied in ATPSs differ in overall composition, namely: (i) 4% PEG 8 000 and 5% dextran 500 000; (ii) 5.6% PEG 8 000 and 7.5% dextran 500 000. The amount of Ab present in both top and bottom phases was determined by analytical protein G chromatography. The partitioning of both anti-CD34-poly(NIPAM) and IgG-poly(NIPAM) was comparable and in both cases the partition coefficients ($K_p$) were higher than 1. The presence of Abs in solution show that the polymer backbone was partially composed of protein units, and, thus the co-polymerization was carried out successfully.

It was observed that for both Abs (anti-CD34 and IgG) the $K_p$ was higher while increasing the concentration of the top phase from 4% to 5.6% (see Figure 19). There was also noted a small effect of salt concentration on the partitioning behavior of poly(NIPAM) conjugates.

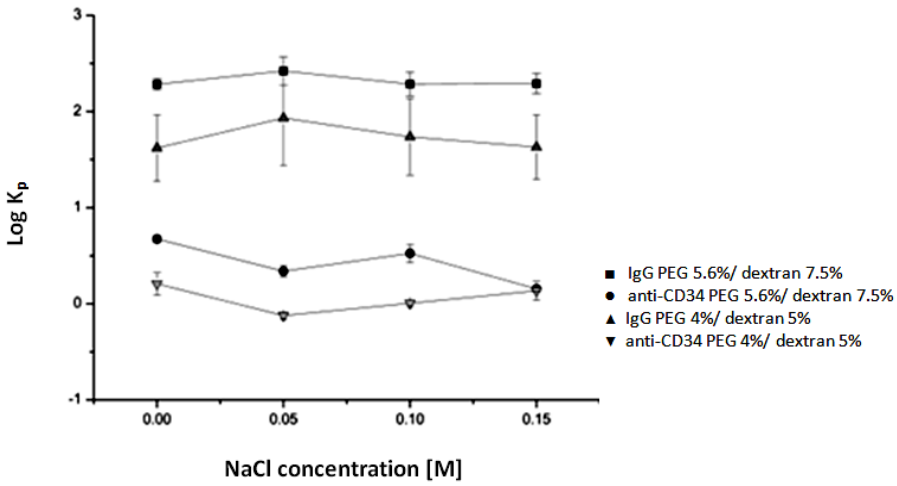


**Figure 19** Log phase of the $K_p$ of both anti-CD34 and IgG antibodies conjugated with poly(NIPAM) in different composition systems (4% PEG 8 000/5% dextran 500 000 and 5.6% PEG 8 000/7.5% dextran 500 000). It is shown that for increasing concentration of the top phase polymer higher values of the $K_p$ are achieved. Results are presented as mean ± SD (standard deviation)(n=2).

From this experiments we can conclude that Ab-poly(NIPAM) bioconjugates achieved both rapid response to changes in temperature and drastic phase separation. The conjugates were conveniently separated from a solution by thermal precipitation, which allowed the selective removal of only the Abs that were conjugated to the smart polymer, poly(NIPAM).

## 4.3. Characterization of monoclonal antibodies against CD34 antigen used as affinity ligands

### *Anti-CD34 partitioning studies*

The partitioning behavior of the Ab-poly(NIPAM) conjugates was studied in ATPSs differ in overall composition, namely: (i) 4% PEG 8 000 and 5% dextran 500 000; (ii) 5.6% PEG 8 000 and 7.5% dextran 500 000. The amount of Ab present in both top and bottom phases was determined by analytical protein G chromatography.

The same phenomena that was observed for ATPSs containing anti-CD34-poly(NIPAM) holded also for systems with "free" anti-CD34, that is, the application of ATPS with a higher tie-line length resulted in a slightly higher $K_p$ (see Figure 20). It was also observed that partitioning of anti-CD34 to the top phase is greater for increasing concentration of NaCl and the highest $K_p$ was obtained for systems with 0.2 M NaCl. It was previously reported in the literature, that at NaCl concentrations above 1 M most proteins tend to favour the upper, PEG rich phase and this was mostly caused by pronounced hydrophobic interactions (Albertsson, 1971). When NaCl is added to the system there is a decrease in the total mass of water, in order to keep constant the final composition of the system in terms of PEG and dextran concentrations. This replacement of water by NaCl can explain the increase in hydrophobicity difference between the phases. Although PEG is weakly hydrophobic, its interactions with hydrophobic domains on proteins may increase at high salt concentrations. In this experiment, the effect was already visible at relatively low concentration of 0.2 M NaCl.

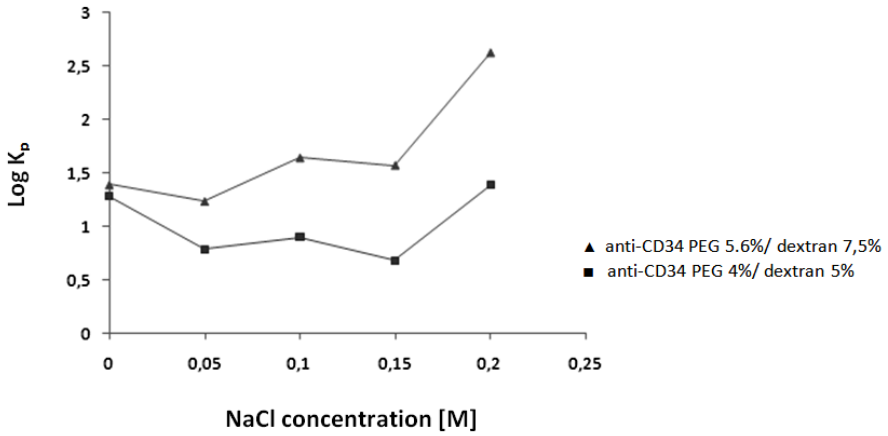


**Figure 20** Log phase of the partition coefficients ($K_p$) of anti-CD34 Abs in different composition systems (4% PEG 8 000/ 5% dextran 500 000 and 5.6% PEG 8 000/7.5% dextran 500 000). It is shown that for increasing concentration of the top phase polymer higher values of the $K_p$ are achieved.

It is also known that the presence of salts creates an electrical potential between the two phases that can drive proteins to one or another phase depending on their net charge (Albertsson, 1971). As it was revealed by isoelectric focusing (see Figure 21) at pH values maintained during the partitioning experiments (pH ranging from 7 to 8) the anti-CD34 remained negatively charged. In an ATPS, anions and cations distribute unequally across the interface. Water structure making ions (e.g. $L^+$, $Na^+$, $NH_4^+$, $Ca^{2+}$, $Mg^{2+}$) favour the more hydrophilic phase (dextran) and water structure breaking ions (e.g. $K^+$, $Rb^+$, $Cs^+$, $Cl^-$, $Br^-$, $I^-$, $SCN^-$) favour the more hydrophobic phase (PEG) (Albertsson, 1971). A slightly lower pH was always noted for the ATPSs with a higher concentration of salt. This occurrence made the proteins less negatively charged and gave rise to weaker interactions between mAb and $Na^+$ ions. The highest partition of mAb to the top phase was noted for the system with the lowest value of pH (pH 7.3), namely for ATPS supplemented with 0.2 M NaCl.

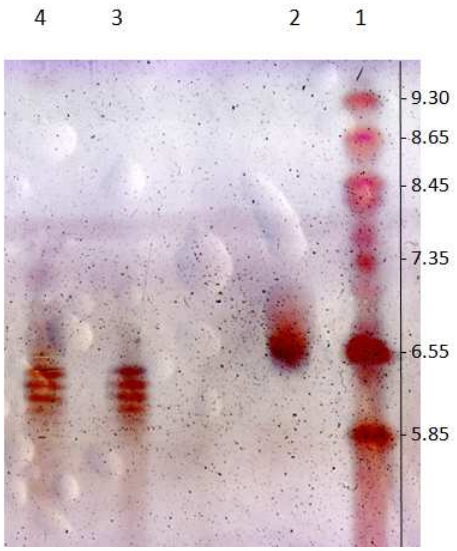


**Figure 21** Silver stained PhastGel IEF 3-9. Lanes ID: 1, isoelectric point standards; 2, anti-CD34 purchased from Biolegend; 3 and 4, anti-CD34 produced by hybridoma cells AC133.1. The isoelectrofocusing of anti-CD34 produced, revealed four bands corresponding to four isoforms with pI ranging from 6.05 to 6.45. It can be also observed that the pI of anti-CD34 from Biolegend is around 6.55.

## 4.4. Expansion and characterization of the model cell lines

Prior to application in ATPSs, $CD34^+$ KG-1a cells and L929 ($CD34^-$) cells were expanded in IMDM culture medium supplemented with 10% FBS and characterized in terms of viability, proliferation and CD34 marker expression.

### *KG-1a cell line as an experimental positive control*

The human $CD34^+$ acute myeloid leukemia cell line (KG-1a) was selected as a simple, positive experimental control. Before application in ATPSs, cells were previously expanded for 9 or 21 days. The viability assessed by trypan blue dye exclusion method was nearly 98% after 1, 3, 6 and 9 days in the culture and remained relatively high even after 21 days of cultivation (96%). Parallel to the viability measurements, cells were also studied using light microscopy. Relatively small, round suspension cells started to create aggregates after 16 days of cultivation (see Figure 22).

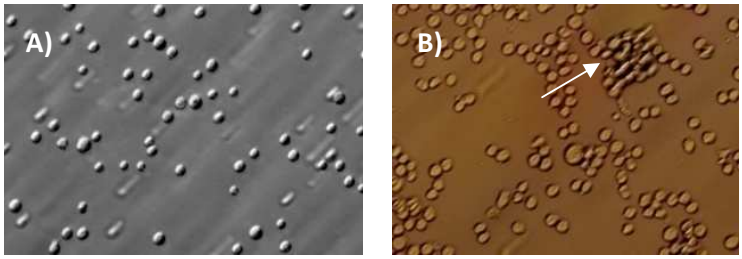


**Figure 22** KG-1a cells in IMDM + 10% FBS after **(A)** 9 days and **(B)** 21 days of cultivation. It is shown that cells are creating aggregates after cultivation longer than 16 days.

The expression of the cell surface marker CD34 was tested after 9 and 21 days respectively, in order to see if there is any correlation between the length of cultivation and the expression level. As expected, cells were $CD34^+$ after 21 days in the culture. From FSC versus SSC plot it was noticed that cells were slightly bigger after 21 days of cultivation in IMDM + 10% FBS (Figure 23).

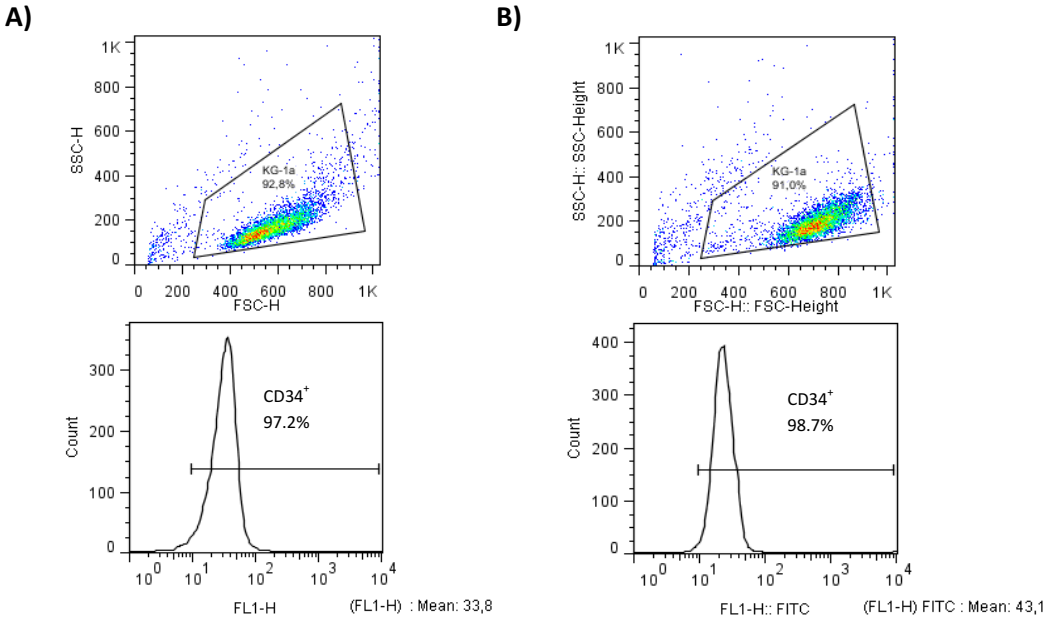


**Figure 23** Flow cytometry analysis of KG-1a cells after **(A)** 9 days and **(B)** 21 days of cultivation in IMDM + 10% FBS. FSC versus SSC plot demonstrates that KG-1a cells are bigger after 21 days of cultivation in culture medium.

### *L929 cell line as an experimental negative control*

The L929 mouse fibrosarcoma cells were expanded for 9 days in IMDM + 10% FBS medium before application to ATPSs. Spindle-shaped L929 cells showed a good proliferative potential, reaching 70-80% confluence after 3-4 days of cultivation (see Figure 24). After 9 days of culture, cells displayed viability of 97%. Since L929 cells were used only as a negative control, CD34 expression was mainly studied during the last day of cultivation, directly before using in further partition experiments (see Figure 25).

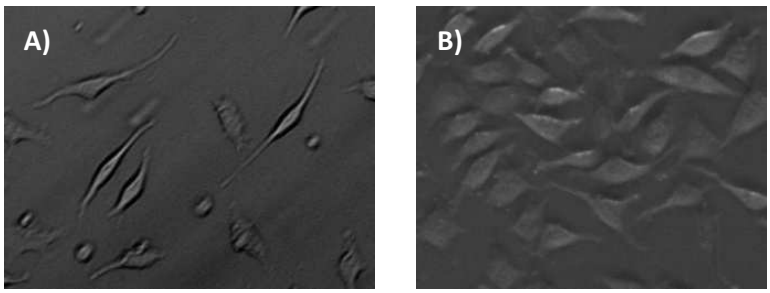


**Figure 24** Spindle-shaped L929 cells after **(A)** 3 days and **(B)** 9 days of cultivation.

L929 cells, as it was revealed by flow cytometry results, were CD34 negative, given to its non-hematopoietic and non-endothelial nature (Theerakittayakorn, et al., 2011). SSC versus FSC showed that L929 cells are more complex than KG-1a cells making these two populations relatively easy to distinguish.

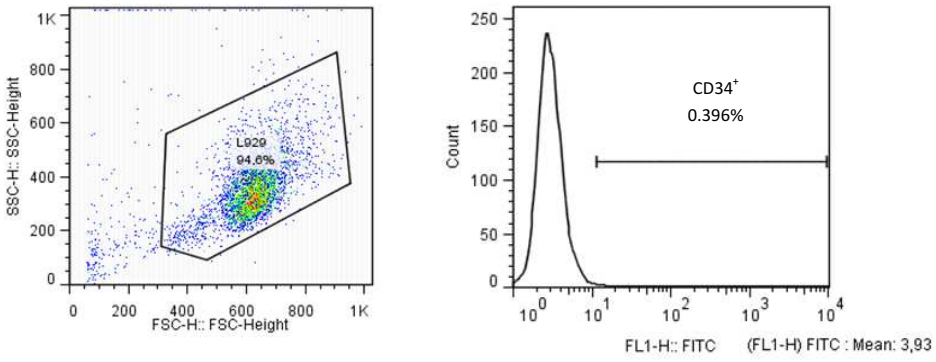


**Figure 25** Flow cytometry analysis of L929 cells after 9 days of cultivation in IMDM + 10% FBS.

## 4.5. KG-1a cells partitioning studies

### *Selection of ATPS suitable for KG-1a cell separation*

Several studies at various PEG and dextran concentrations using PEGs of different MW have been performed in order to select the best system for selective partition of KG-1a cells. Experiments in the absence and presence of affinity ligands were carried out using the same conditions for all systems that were being examined. The effect of increasing concentration of salt (NaCl) was also evaluated.

### *Non-affinity partitioning of KG-1a cells*

The same general trend that was previously described in the literature (Kepka, 2004) for the proteins, holded for KG-1a cells, the higher the MW of one polymer, the lower partition to the polymer-rich phase. In the systems without the addition of affinity ligands, cells were pushed upwards if lower MW PEG was replaced with higher MW PEG, and the highest value of $K_p$ was noted for the system PEG 3 000/ dextran 500 000 with no addition of salt. In the absence of NaCl, more than 60% cells were distributed in the top phase in all examined systems. However, increasing the concentration of NaCl, the $K_p$ of the cells decreased and the lowest amount of KG-1a cells in the PEG-rich phase was observed for 0.2 M NaCl (see Figure 26).

### *Affinity partitioning of KG-1a cells*

ATPSs containing affinity ligands were also tested. Both for systems containing anti-CD34-poly(NIPAM) and only purified anti-CD34, the $K_p$ was higher when the affinity ligands were present in the systems. There was a marked difference in partitioning behavior of cells between the systems with anti-CD34-poly(NIPAM) and "free" anti-CD34. While in systems with Ab-poly(NIPAM) conjugates the increasing concentration of salt pushed the cells to the bottom phase, there was no significant difference between percentages of the cells partitioned to the top phase in the ATPS with "free" anti-CD34 in the absence and presence

of salt. The $K_p$ of the monoclonal anti-CD34 was rising with increasing concentration of salt (see 4.3. Characterization of monoclonal antibodies against CD34 antigen used as affinity ligands), and the anti-CD34 attracted cells to the upper phase. At the same time, however, applying of higher concentration of salt pushed the cells to the bottom phase. The inverse effects resulted with almost the same partitioning of the cells to the top phase when anti-CD34 were added as affinity ligands to the ATPSs with varying concentration of salt.

Theoretically, the cells should be distributed in the bottom phase in the presence of more than 0.1 M NaCl (Kumar, et al., 2001). However, specific interactions between the cells and the affinity-ligands that are preferentially partitioned to the top phase allowed the cells to move to the PEG-rich phase.

A PEG 8 000 (4%)/ dextran 500 000 (5%) was selected as a basal ATPS suitable for cell separation for both systems: containing the temperature sensitive polymer, poly(NIPAM), used as ligand carrier, as well as for systems with only "free" Abs anti-CD34. The choice was based on the most marked difference between the $K_p$ of cells in the presence and absence of affinity ligands (see Figure 26).

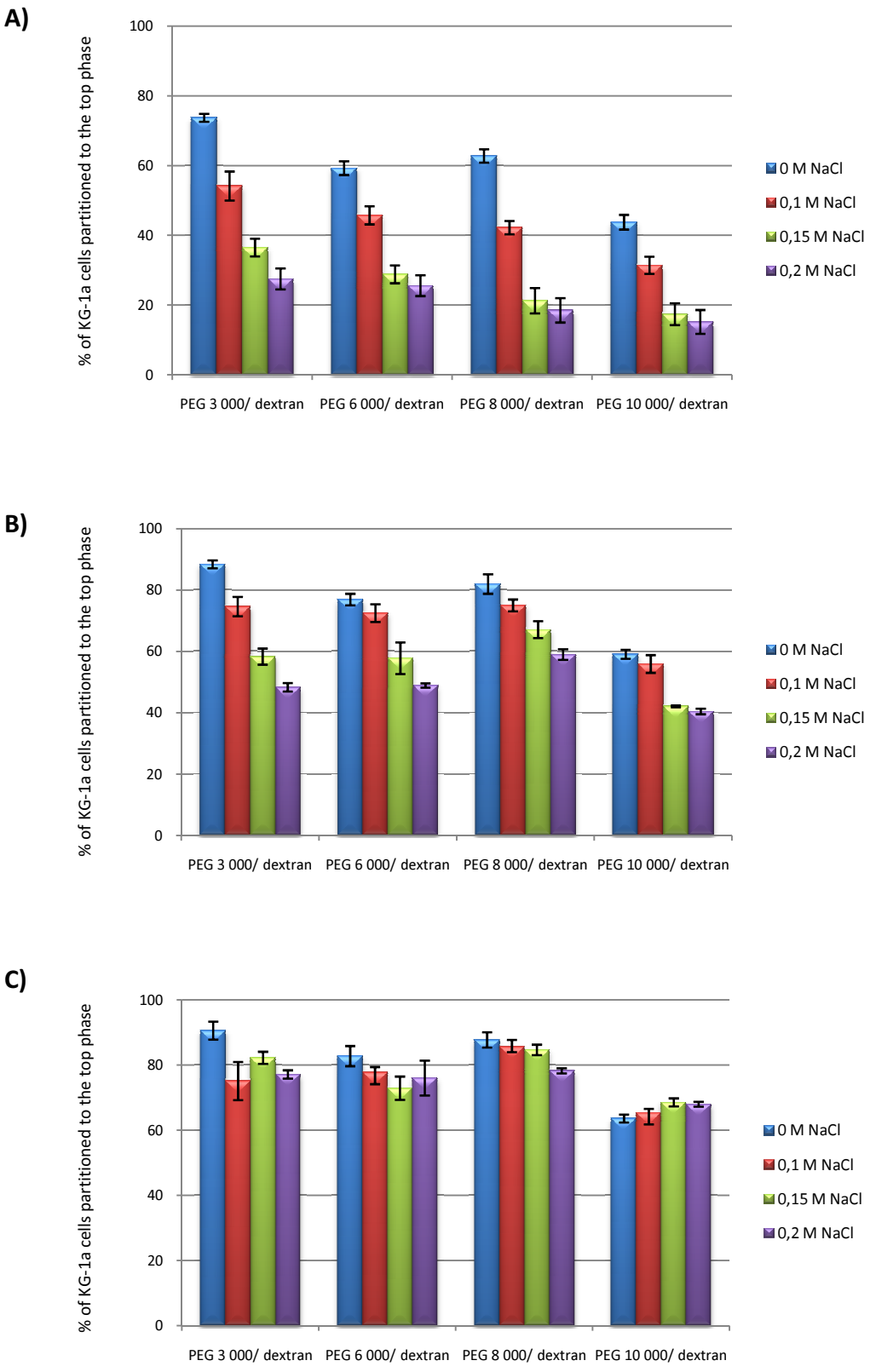


**Figure 26** Partitioning of KG-1a cells in ATPSs differ in PEG MW and NaCl concentration **(A)** in the absence of affinity ligands, and in the present of the affinity ligands **(B)** anti-CD34-poly(NIPAM) conjugates and **(C)** mAb anti-CD34 respectively. Results are presented as mean ± SD (standard deviation) (n=3).

One way to alter the partitioning of molecules in ATPSs is to increase the tie-line length (Walter, et al., 1994). This phenomena is mainly caused by the fact that polymer phases differ more in composition at higher concentrations than close to the binodal (Albertsson, 1971) (Forciniti, et al., 1991). Taking this dependence into account the comparison between two systems with different polymer concentrations was performed. Two ATPSs comprised of 4% PEG /5% dextran and 5.6% PEG /7.5% dextran respectively, were studied in order to determine the influence of increasing polymer concentration on the partitioning of cells along with different NaCl concentrations. The same conditions with different salt concentration (from 0 to 0.2 M) were used for both types of affinity ligands. The application of ATPS with a higher tie-line length (higher concentration of polymers) gave a lower $K_p$ of KG-1a cells in the absence of affinity ligands. However, in the presence of both affinity ligands (anti-CD34-poly(NIPAM) conjugates and "free" anti-CD34), the partitioning of cells to the top phase was slightly higher. The effect of the PEG 8 000 and dextran 500 000 concentration on the partitioning is summarized in the Table 3. The same trend for increasing salt concentration previously described for systems with poly(NIPAM) conjugates, was observed for both systems, namely for 4% PEG/ 5% dextran and 5.6% PEG/ 7.5% dextran. The high selectivity for systems containing 0.15 M NaCl is in accordance with previous experiments.

**Table 3** Partitioning of KG-1a in PEG 8 000/ dextran 500 000 at different polymer concentrations. Results are presented as mean ± SD (standard deviation)(n=2).

| NaCl (M) | Percentage of cells partitioned to the Top Phase (%) | | | | | |
|---|---|---|---|---|---|---|
| | No-affinity ATPS | | Affinity ATPS | | | |
| | | | Anti-CD34-poly(NIPAM) | | Anti-CD34 | |
| | 4% PEG / 5% dextran | 5.6% PEG / 7.5% dextran | 4% PEG / 5% dextran | 5.6% PEG / 7.5% dextran | 4% PEG / 5% dextran | 5.6% PEG / 7.5% dextran |
| 0 | 63±2 | 61±2 | 82±6 | 85±1 | 88±1 | 81±14 |
| 0.1 | 43±4 | 39±8 | 75±3 | 78±7 | 86±2 | 87±2 |
| 0.15 | 30±7 | 22±4 | 67±5 | 71±2 | 85±7 | 90±8 |
| 0.2 | 25±7 | 21±1 | 59±2 | 61±6 | 78±8 | 80±9 |

Based on these results, it is reasonable to assume that a two-phase system composed of 5.6% PEG 8 000 / 7.5% dextran 500 000 supplemented with 0.15 M NaCl and with an adequate affinity ligand can be expected to be suitable for the specific separation of $CD34^+$ cells to the top phase. Thus, the selected ATPS may constitute a promising separation option for HSC selection, since it was previously reported in the literature that 4% PEG 6 000/ 5% dextran 500 000 ATPS with 0.15 M NaCl concentration gathered almost all human erythrocytes (major contaminant cells) in the bottom phase (Albertsson, 1971).

### *The influence of the KG-1a cell cultivation length on the partitioning behavior of cells*

The selected ATPS, namely 5.6% PEG 8 000/ 7.5% dextran 500 000, was used for further studies with the KG-1a cell line. KG-1a cells prior to the application to ATPSs, as it was previously described, were cultivated for 9 or 21 days, respectively (see 4.4. Expansion and characterization of the model cell lines). Systems with both types of affinity ligands were examined in order to estimate if there is any influence of the KG-1a cells cultivation length on the partitioning behavior of cells. Results revealed that there was a slight difference in the distribution of cells that were cultivated for a longer period of time. The partitioning to the top phase was lower in case of KG-1a cells that were cultivated for 21 days, both for systems containing anti-CD34-poly(NIPAM) conjugates or "free" anti-CD34 (see Table 4). This phenomena was previously reported in the literature while describing partitioning behavior of K-562 cells (human cell line originally derived from a patient with chronic myelogenous leukemia in blast crisis) associated with different stages of culture growth (Walter, et al., 1987). The decrease in mean partition ratio of the cell population as a whole during culture was attributed to the lower partition ratio of non-viable cells which increase with time of culture. Since the viability of KG-1a cells remained almost the same during the whole time of cultivation, the change in partitioning behavior may be explained by observed size/diameter increase and potentially, by tendency to create aggregates after 16 days of culture in IMDM + 10% FBS. However, the basis for this phenomenon still remains unclear and further studies are necessary.

**Table 4** Partitioning of KG-1a cells using 5.6% PEG 8 000/ 7.5% dextran 500 000 ATPS system. Before selection in ATPS the cells were cultivated in IMDM + 10% FBS culture medium for 9 or 21 days respectively. Results are presented as mean ± SD (standard deviation)(n=2).

| NaCl (M) | Percentage of cells partitioned to the Top Phase (%) | | | | | |
|---|---|---|---|---|---|---|
| | No-affinity ATPS | | Affinity ATPS | | | |
| | 9 days of cultivation | 21 days of cultivation | Anti-CD34-poly(NIPAM) | | Anti-CD34 | |
| | | | 9 days of cultivation | 21 days of cultivation | 9 days of cultivation | 21 days of cultivation |
| 0 | 61±2 | 60±4 | 85±1 | 78±4 | 81±14 | 77±9 |
| 0.1 | 39±8 | 34±3 | 78±7 | 62±14 | 87±2 | 72±9 |
| 0.15 | 22±4 | 18±3 | 71±2 | 59±4 | 90±8 | 88±9 |
| 0.2 | 21±1 | 18±1 | 61±6 | 35±7 | 80±9 | 77±13 |

## 4.6. KG-1a cell morphology studies after partitioning in ATPS

The morphology of KG-1a cells after partitioning in 5.6% PEG 8 000/ 7.5% dextran 500 000 ATPS was further studied in order to determine the influence of affinity ligands, the phase forming polymers (PEG and dextran) and increasing concentration of NaCl. Two parallel partitioning experiments with both type of affinity ligands, anti-CD34-poly(NIPAM) and anti-CD34, were performed and cells were further cultivated and studied for 7 days.

### *KG-1a cells morphology studies after affinity partition in ATPS with "free" anti-CD34*

More than 80% of cells remained viable directly after partition experiments in ATPSs containing "free" anti-CD34 mAbs as affinity ligands. The viability of cells was slightly decreasing while using salt concentrations above 0.3 M NaCl and reached the lowest percentage of living cells (82%) for 0.5 M NaCl concentration. Cells maintained nearly the same percentage of viability even after 7 days of cultivation.

Light microscopy observations showed that majority of the cells were cell suspended (i.e. not clumped) after 3 days in a culture and at this level no obvious morphological differences were visible. After 5 days of culture, cells started to create aggregates and the effect was more explicit for higher salt concentrations (see Figure 27).

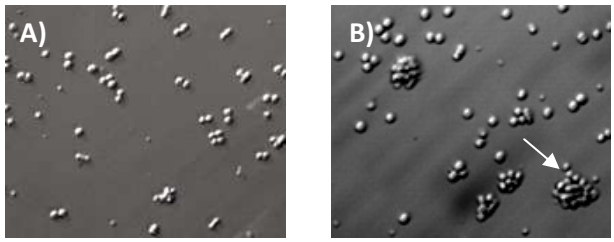


**Figure 27** KG-1a cells after partitioning in 5.6% PEG 8 000/ 7.5% dextran 500 000 ATPS with **(A)** 0 M and **(B)** 0.3 M NaCl after 5 days of cultivation in IMDM + 10% FBS.

Immunophenotype analysis was done before and after 7 days of culture. No significant effect on the CD34 expression was observed. Approximately 99% of cells remained CD34 positive after partitioning, as well as, after 7 days in culture (see Figure 28). FSC versus SSC plots, that provide information about cell size and granularity, confirm that cell size and complexity remained unchanged after partitioning in ATPS in the absence of NaCl. The same phenomena was observed for all ATPSs that were supplemented with less than 0.5 M NaCl. The most harsh effect on cells was noted for system containing 0.5 M NaCl, what can be observed from FSC versus SSC plots presenting changed population of cells (see Figure 28, C and E)

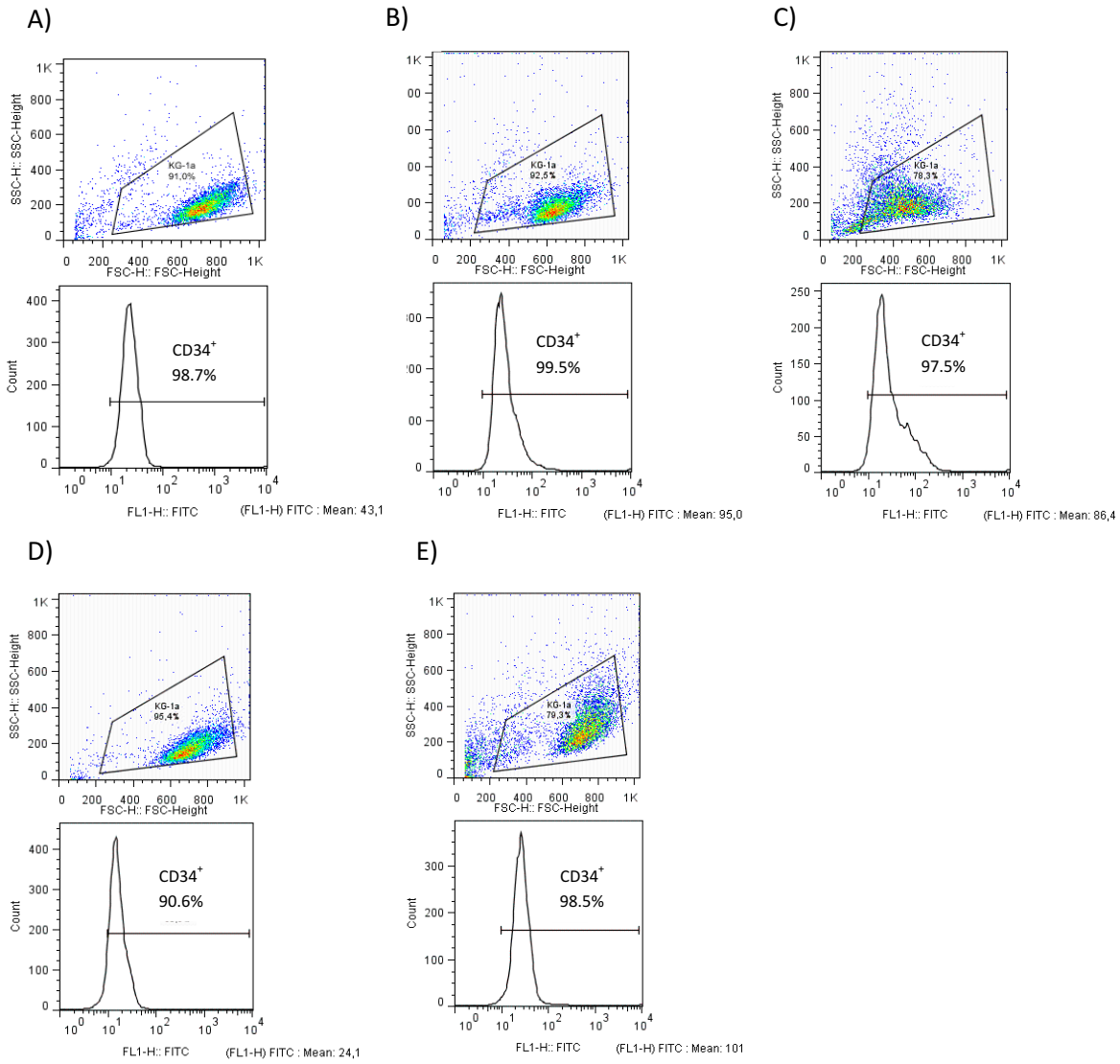


**Figure 28** Flow cytometry analysis for the expression of CD34 antigen of KG-1a cells collected from the interface after partitioning in 5.6% PEG/ 7.5% dextran ATPS. Cells were subsequently cultured for 7 days in IMDM + 10% FBS culture medium. Population of KG-1a cells **(A)** before partitioning and directly after partitioning in ATPSs containing anti-CD34 as affinity ligands in the presence of: **(B)** 0 M NaCl and **(C)** 0.5 M NaCl, and after 7 days in the culture medium: cells derived from the ATPS with **(D)** 0 M NaCl and **(E)** 0.5 M NaCl.

### *KG-1a cell morphology studies after affinity partition in ATPS with anti-CD34-poly(NIPAM) conjugates*

The viability of cells directly after partition experiment reached values of approximately 70%, that was slightly lower than the viability obtained for the systems

containing “free” anti-CD34 Abs. As expected, the cells displayed good proliferative potential. It is believed that poly(NIPAM) caused no harmful effect on cells.

However, after 5 days of culture the effect of aggregation was noticeable and at this level there were no considerable morphological difference between cells derived from ATPS tubes with different salt concentration (0 to 0.2 M NaCl). The aggregation progressed and numerous clumps were visible after 5 (see Figure 29) and 7 days in the culture using IMDM + 10% FBS. Since the highest concentration of salt used in the experiment was 0.2 M, there was no significant difference between cells derived from different ATPSs.

As expected, after 7 days, cells were expressing CD34 marker.

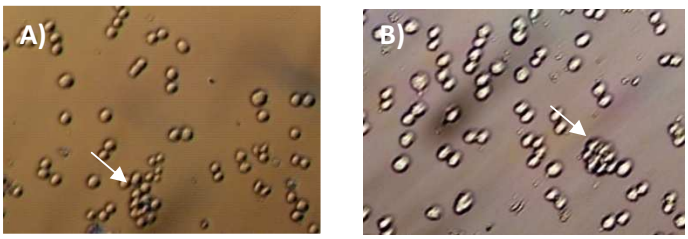


**Figure 29** KG-1a cells after partitioning in 5.6% PEG 8 000/ 7.5% dextran 500 000 ATPS with **(A)** 0 M and **(B)** 0.2 M NaCl after 5 days of cultivation in IMDM + 10% FBS. It is demonstrated that cells are starting to aggregate after 5 days of cultivation.

## 4.7. Specific separation of KG-1a cells from L929 cells

As a model system, the specific separation of KG-1a cells ($CD34^+$) from L929 cells ($CD34^-$) was investigated using anti-CD34-poly(NIPAM) conjugates and anti-CD34 affinity ligands, respectively. KG-1a and L929 cells were mixed at different proportions and applied to the 5.6% PEG 8 000/ 7.5% dextran ATPS selected in previous experiments. After separation cells that partitioned to the top phase were collected at interface by centrifugation at 180*xg* for 5min. All experiments were performed with a constant total cell number of 1.75 x $10^6$ cells.

### *Separation of KG-1a cells from L929 cells in affinity ATPSs with anti-CD34*

The two types of viable cells can be identified in flow cytometry analysis, which shows two different fluorescent peaks (Figure 30). The peak height is correlated to the viable cell, the first corresponding to $CD34^-$ cells (L929) and the second to the $CD34^+$ cells (KG-1a).

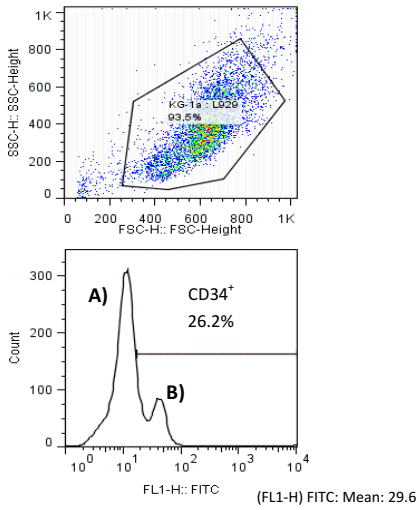


**Figure 30** Flow cytometry analysis of KG-1a and L929 cells. **(A)** L929 and **(B)** KG-1a cells at ratio of 70:30 were applied.

As seen in Figure 31, the percentage of $CD34^+$ in the top phase (collected from the interface after centrifugation) was relatively high in all examined cases. The lowest amount of $CD34^+$ cells (79%) was achieved when KG-1a : L929 cell ratio of 20:80 was used. Since the L929 cell proportion was higher in the cell mixture, the KG-1a cells were more contaminated with L929 cells in the top phase. In all other cases the percentage of CD34 positive cells in the PEG phase amounted more than 80% and there was a slight increase correlated with a higher ratio of KG-1a cells in the cell mixture. The maximum value reached 86% of $CD34^+$ cells partitioned to the top phase when KG-1a cells comprised 80% of the cell mixture. KG-1a cells preferentially partitioned to the top phase and the number of $CD34^+$ cells in the PEG phase was increased when KG-1a cells were present at higher percentages in the initial population.

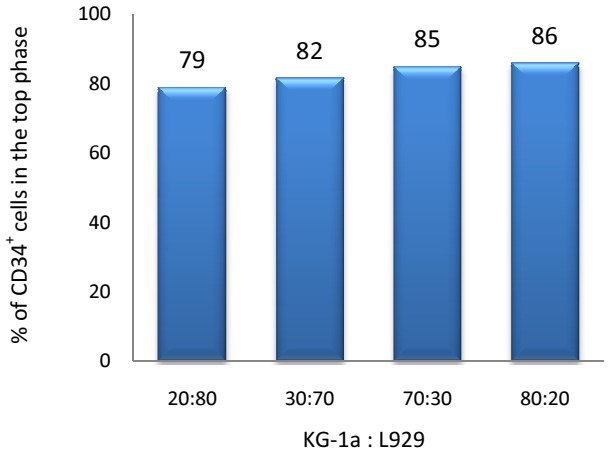


**Figure 31** The percentage of $CD34^+$ cells in the top phase after specific separation of the mixture of KG-1a and L929 cells. The percentage values were estimated by flow cytometry analysis of samples collected from the Interface after centrifugation at 180*xg* for 5 min.

It was also observed that lower purification was achieved when KG-1a cells were applied to the ATPSs at higher ratios (see Table 5). This may indicate that the amount of anti-CD34 (10 μg) , used as affinity ligands, was not enough for the complete separation of a greater number of cells. Although the highest PF was obtained when KG-1a : L929 cells were applied at 20:80 ratio, the %R of $CD34^+$ was slightly lower in comparison to the runs with 70% and 80% of KG-1a cells in the initial population.

The more complete separation of KG-1a from L929 cells could be probably possible when applying additional extraction steps.

**Table 5** Quantification of $CD34^+$ cell recovery and purification factor achieved after specific separation of KG-1a and L929 cell mixture in the ATPS composed of 5.6% PEG 8 000 and 7.5% dextran 500 000 with "free" anti-CD34 as affinity ligands.

| KG-1a : L929 | Cell Recovery [%] | Purification Factor |
|---|---|---|
| 20 : 80 | 69 | 4.0 |
| 30 : 70 | 56 | 2.7 |
| 70 : 30 | 76 | 1.2 |
| 80 : 20 | 85 | 1.1 |

### *Separation of KG-1a cells from L929 cells in affinity ATPS with anti-CD34-poly(NIPAM) conjugates*

Figure 32 demonstrates the distribution of KG-1a cells after partitioning of KG-1a and L929 cells at different ratios in the 5.6% PEG 8 000/ 7.5% dextran 500 000 ATPS. When anti-CD34-poly(NIPAM) conjugates were used, the distribution of the KG-1a cells to the top phase was significantly lower than in the systems containing "free" Abs and achieved the maximum value when KG-1a: L929 ratio was 80:20. The same general trend that was previously noted for systems containing anti-CD34 as affinity ligands, holded also for ATPSs containing anti-CD34-poly(NIPAM) conjugates, that is, the higher ratio of KG-1a cells in the mixture of cells, the greater number of $CD34^+$ cells partitioned to the PEG rich phase. The percentage of $CD34^+$ cells present in the top phase was estimated by flow cytometry analysis as shown in Figure 32. Although it was not possible to obtain the complete separation (Kumar, et al., 2001), the values up to 57% cells partitioned to the top phase were achieved when KG-1a : L929 cells were applied to the ATPS at the 80:20 ratio.

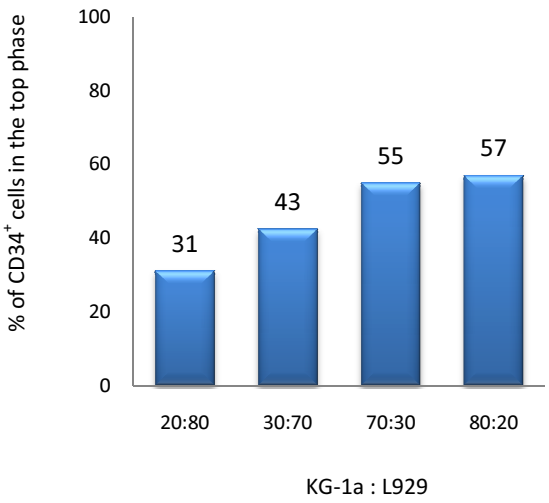


**Figure 32** The percentage of $CD34^+$ cells in the top phase after specific separation of the mixture of KG-1a and L929 cells. The percentage values were estimated by flow cytometry analysis of samples collected from the interface after centrifugation at 180*xg* for 5 min.

Since cells collected from the top phase were highly contaminated with $CD34^-$ cells, we may draw a conclusion that there are some unspecific interactions between L929 cells and poly(NIPAM) polymer. Moreover, the decreasing PF with increasing ratio of L929 present in the cell mixture may lead us to assumption, that the amount of Ab conjugated with poly(NIPAM) polymer was not enough for the complete separation and there are more separation steps needed in order to obtain better results (see Table 6). Although some purification was achieved (~2 fold) when KG-1a cells were applied to the ATPS at lower ratios, the recovery percentage was significantly lower in comparison to the runs with higher number of KG-1a cells present in the initial population.

**Table 6** Quantification of $CD34^+$ cell recovery and purification factor achieved after specific separation of KG-1a and L929 cell mixture in the ATPS composed of 5.6% PEG 8 000 and 7.5% dextran 500 000 with anti-CD34-poly(NIPAM) conjugates as affinity ligands.

| KG-1a : L929 | Cell Recovery [%] | Purification Factor |
|---|---|---|
| 20 : 80 | 10 | 1.6 |
| 30 : 70 | 32 | 1.4 |
| 70 : 30 | 42 | 0.8 |
| 80 : 20 | 46 | 0.7 |

## 4.8. Processing of UCB samples

On the basis of previous results, systems composed of 5.6% PEG 8 000 and 7.5% dextran 500 000 supplemented with 0.15 M NaCl and affinity ligands were examined for the specific separation of hHSC from UCB. Two different experiments were performed in order to select the best conditions for hHSC separation from UCB.

### *Trial 1*

Affinity ATPSs containing poly(NIPAM) conjugated with anti-CD34 and unconjugated mAb anti-CD34 were tested for the selective separation of hHSCs. ATPSs with Gammanorm IgG-poly(NIPAM) conjugates and Gammanorm IgG "free" Abs, respectively, were also performed in order to confirm the specificity of target Abs (anti-CD34) in both forms, conjugated and free.

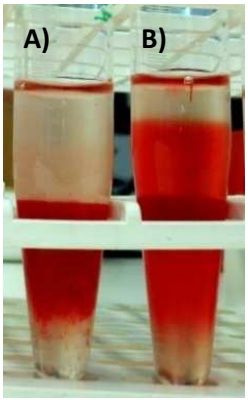


**Figure 33** Photography of 5.6% PEG 8 000 – 7.5% dextran 500 000 ATPS containing UCB samples and "free" Abs as affinity ligands: **(A)** anti-CD34 and **(B)** IgG. The picture was taken after cells settling and partitioning. It is shown that most of the contaminant cells (RBC) were partitioned to the bottom phase.

In general, cells partition to the volume between one of the phases and the interface. As expected, most of the contaminant cells, namely erythrocytes, partitioned to the more hydrophilic dextran-rich phase at 0.15 M NaCl concentration (Albertsson, 1971), as it can be seen on the picture (Figure 33).

After cell collection, the number of cells from each phase was determined by trypan blue exclusion method and the flow cytometry results revealed if the partition was selective

(see Figure 34). Starting from a population of 0.11% $CD34^+$ cells it was possible to achieve values up to 18% stem/progenitor cells using thermosensitive polymer copolymerized with the target Abs. The partitioning of the HSCs to the top phase was possible due to the specific interactions between $CD34^+$ cells and the Ab.

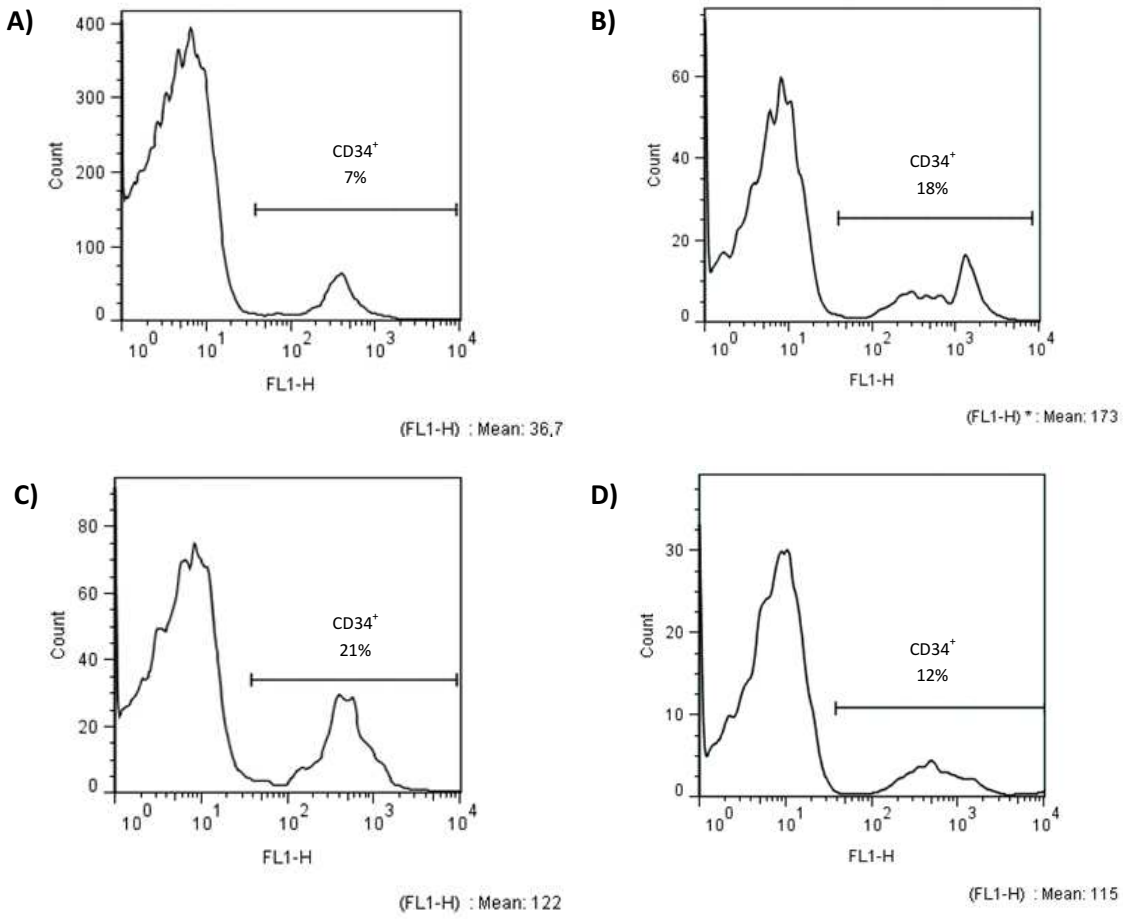


**Figure 34** Flow cytometry analysis for the expression of CD34 antigen of cells collected from the interface of ATPS, after settling and partitioning in the presence of respectively: **(A)** IgG-poly(NIPAM), **(B)** anti-CD34-poly(NIPAM), **(C)** IgG, **(D)** 5 µg of anti-CD34. Prior to flow cytometry analysis cells were stained with the mouse anti-human mAbs (FITC conjugated) against CD34.

Relatively high values of $CD34^+$ cells (21%) obtained for systems containing Gammanorm IgG may give evidence of some unspecific interactions between polyclonal IgG and cells (Figure 34 C). Since it was previously reported (Rosa, et al., 2007) that IgG has an pI around 9, it means that the biomolecule is positively charged at pH values maintained during

the experiment. Mammalian cells have a negative overall charge at a pH of around 7.0 (Walter, et al., 1982) (Albertsson, 1971), what may indicate some electrostatic interactions between cells and IgG.

Taking into account the expression of CD34 in the initial cell sample (0.11%, non-fractioned, whole UCB) it was possible to determine PFs (see Table 7). The high value was obtained for the system with anti-CD34-poly(NIPAM) as affinity ligand emphasizing the efficiency of the designed ATPS.

Another observation was a significant PF in the system containing only Gammanorm IgG. From this we can conclude, that for the specificity determination, it is necessary to perform a control run in the absence of any Abs.

Although significant purification was also achieved (64 fold) using poly(NIPAM) conjugated with Gammanorm IgG, the percentage of $CD34^+$ recovered cells is lower when comparing to the experiment with anti-CD34-poly(NIPAM).

**Table 7** Quantification of $CD34^+$ cell recovery and purification factor achieved after UCB partitioning in the ATPS composed of 5.6% PEG 8 000 and 7.5% dextran 500 000.

| | Cell Recovery [%] | Purification Factor |
|---|---|---|
| IgG- poly(NIPAM) | 7 | 64 |
| anti-CD34-poly(NIPAM) | 36 | 164 |
| IgG | 7 | 190 |
| anti-CD34 (5 µg) | 9 | 109 |

### *Trial 2*

Systems with the same properties as used in the previous experiment (see Trial 1 section) with different affinity ligands, namely poly(NIPAM) conjugated with mAb anti-CD34 and "free" anti-CD34, were prepared in order to select the best conditions for hHSC separation and confirm earlier results. ATPS with unconjugated poly(NIPAM) was also studied to determine if there are any interactions between the thermosensitive polymer and cells. A control run was performed, where cells were added to ATPS, in a 0.01 M PBS solution in the absence of affinity ligand. Flow cytometry analysis was performed using indirect labeling (the goat anti-mouse IgG (H+L), Alexa Fluor 488 – conjugated Abs for anti-CD34) for systems containing "free" anti-CD34 as affinity ligands and direct labeling using the mouse anti-human mAbs (FITC) against CD34 for other systems. Results are shown in the Figures 36-37.

The same phenomena as noted before, holded also for this experiment, that is, most of the cells partitioned to the bottom phase (see Figure 35). Since the vast majority of the UCB cells are RBC, the main contaminants, the effect of extremely low $K_p$ was expected.

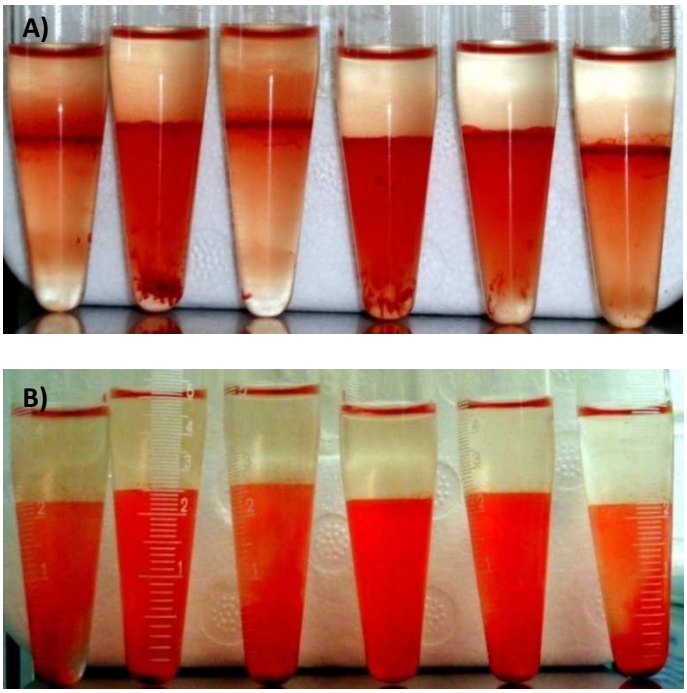


**Figure 35** Photographs of 5.6% PEG 8 000/ 7.5% dextran 500 000 ATPSs containing UCB sample after partitioning process: **(A)** before centrifugation and **(B)** after centrifugation at 180*xg* for 5 min. The cells from the interface were collected and further analyzed.

As expected, the lowest percentage of $CD34^+$ cells was noted for the system without any affinity ligand (see Figure 36 A). The amount of stem/progenitor cells collected from the system with unconjugated poly(NIPAM) may stress that there are some unspecific interactions between cells and the polymer. High values of positive cells were obtained when cells prior to ATPS partitioning were incubated with mAbs anti-CD34 (see Figure 37 B and C). It was also noted that for increased amount of the target Ab (anti-CD34) the percentage of stem/progenitor cells is higher, reaching the value of 41% $CD34^+$ cells when 10 μg of anti-CD34 was used, emphasizing the selectivity of the designed system. Moreover, it was observed that for runs with "free" anti-CD34 as affinity ligands, the values of positive cells are significantly higher when compared to the results obtained in the previous experiment (see Trial 1 section). This indicates that affinity interactions between "free" anti-CD34 did in fact occur and cells were still labeled with primary Abs after recovery from the ATPSs. Thus, in order to obtain the real number of positive cells in the experiments with "free" mAb as affinity ligand, it is crucial to apply indirect labeling.

Taking into account the expression of CD34 in the initial sample (0.17%, non-fractioned, whole UCB) it was possible to determine PFs (Table 8). Significant values were achieved for ATPSs with "free" mAbs anti-CD34. An appreciable difference was noted when the amount of Ab was increased from 5 μg to 10 μg and reached values of PFs were respectively: 6 and 13 times higher from values obtained in the control run.

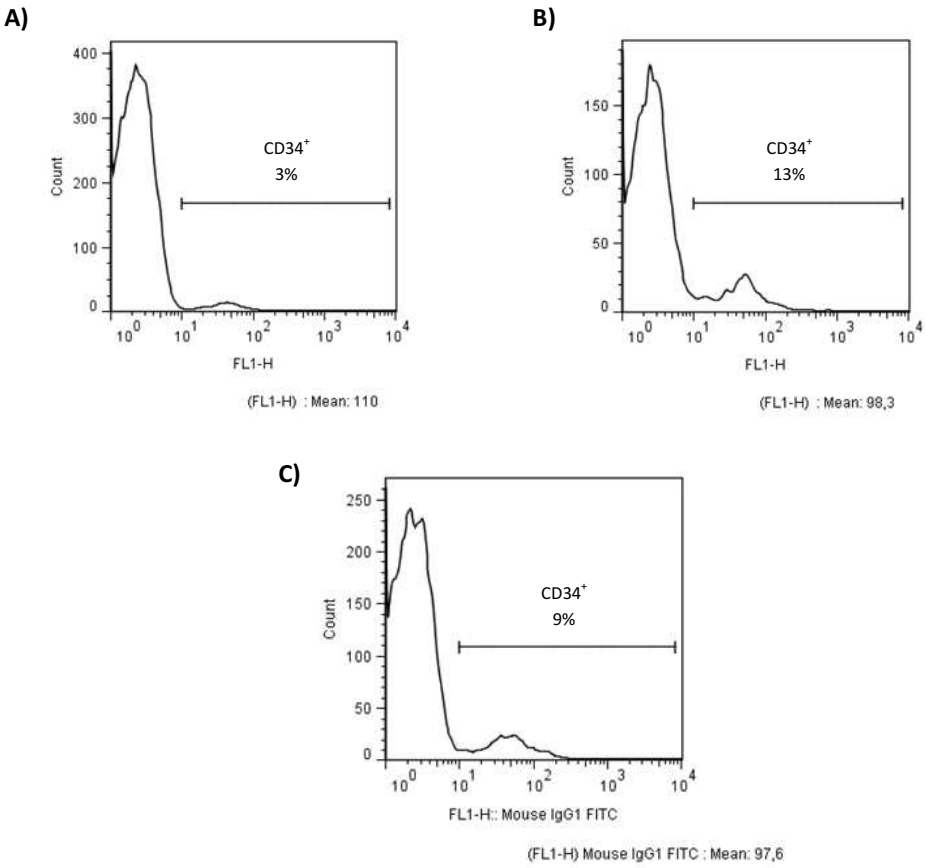


**Figure 36** Flow cytometry analysis for the expression of CD34 antigen of cells collected from the interface of ATPS, after settling and partitioning in the presence of respectively: **(A)** PBS (control run) **(B)** poly(NIPAM) **(C)** anti-CD34-poly(NIPAM). Cells were stained with the mouse anti-human mAbs (FITC conjugated) against CD34.

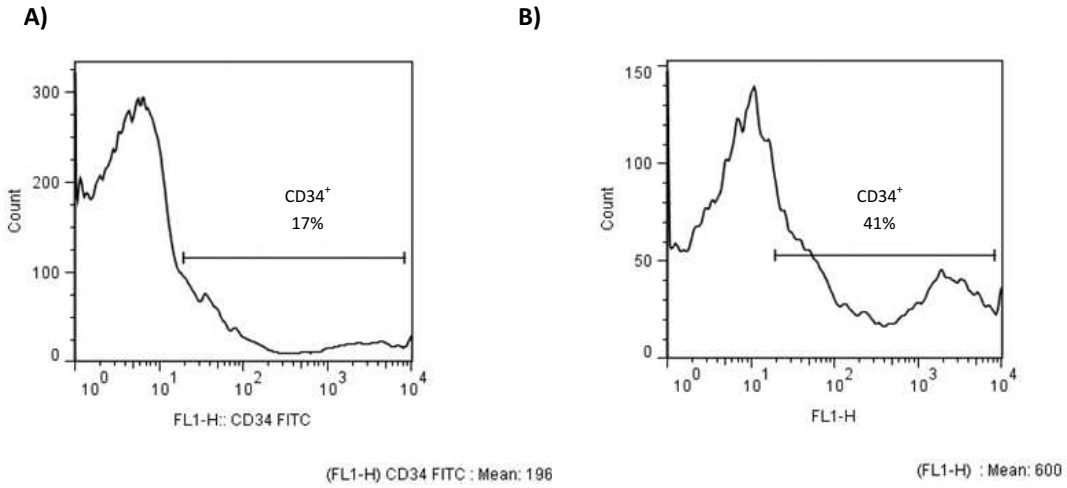


**Figure 37** Flow cytometry analysis for the expression of CD34 antigen of cells collected from the interface of ATPS, after settling and partitioning in the presence of respectively: **(A)** 5 µg anti-CD34 and **(B)** 10 µg anti-CD34. Cells were stained with the goat anti-mouse IgG (H+L), (Alexa Fluor 488 - conjugated) Abs for anti-CD34.

For any cell separation procedure, a balance must be struck between the purity and the recovery of the desired cells. Steps to increase the cell purity should be easy to accomplish. The easiness of recovering the desired cells after partitioning constitutes one of the greatest achievements of the ATPS. Although some purification was achieved in the absence of affinity ligands (18 fold), the recovery of $CD34^+$ cells is relatively low when comparing to the other ATPSs that were studied. Extremely high values (93%) of recovered cells were obtained for system containing 10 μg of anti-CD34. Although, relatively high percentage of positive cells was achieved for the ATPS with unconjugated poly(NIPAM), the value of cell recovery was very low (only 4%), indicating that in order to obtain higher selectivity the copolymerization with target Abs is crucial.

**Table 8** Quantification of $CD34^+$ cell recovery and purification factor achieved after UCB partitioning in the ATPS composed of 5.6% PEG 8000 and 7.5% dextran 500 000.

| | Cell Recovery [%] | Purification Factor |
|---|---|---|
| control | 6 | 18 |
| poly(NIPAM) | 4 | 76 |
| anti-CD34-poly(NIPAM) | 19 | 53 |
| anti-CD34 (5 μg) | 54 | 100 |
| anti-CD34 (10 μg) | 93 | 241 |

# 5. CONCLUSIONS AND FUTURE TRENDS

ATPS offer extremely simple and powerful media for separation. Not only the engineering aspects of extraction in ATPS are presenting no obstacles, but also the method is well adapted for the use with commercially available equipment (Hatti-Kaul, 2000). ATPS consisting of PEG and dextran polymers constitute a mild and non-toxic environment suitable for cell separation.

The present work aimed at the optimization of a novel separation method based on ATPS to isolate and purify human HSC from UCB and the main goal was successfully achieved.

The synthesis of the Ab-poly(NIPAM) conjugates was carried out successfully and from the thermoprecipitation and Protein G quantification results we can conclude that the conjugation-polymerization does occur. However, additional tests with different proportions of Abs, itaconic anhydride and NIPAM would be desirable to obtain a better insight into the best reaction conditions. The affinity properties of the Abs may have been diminished during the conjugation step with itaconic anhydride, which could have possibly occurred on a functional groups of the Abs. This phenomena may partially explain the difference observed in the experiments in the presence of both types of affinity ligands, namely anti-CD34-poly(NIPAM) and "free" anti-CD34.

Preliminary tests with KG-1a cells revealed that $K_p$ is remarkably higher when the affinity ligands are present in the ATPSs. These results were expected, since Protein G quantification tests showed that both types of affinity ligands are preferably partitioned to the top phase. Affinity interactions between cells and ligands have been obviously responsible for the observed phenomena. Slightly better results were obtained for the systems containing only "free" anti-CD34. Although highly specific immunoligands, mAb anti-CD34, show greater potential, from a process economic point of view they are still considered less advantageous. The successful recycling of such costly affinity ligands constitutes a great approach for the preparative application.

KG-1a cell morphology studies after partitioning in ATPSs revealed that both constituent polymers and affinity ligands have no harmuful effect on cells. However, too high concentration of salt may decrease the viability of cells, what have been noted for systems with 0.5 M NaCl.

The preliminary experiments let to the selection of the prospective affinity ATPS possessing appropriate features for the specific HSC separation, namely a two-phase system composed of 5.6% PEG 8 000 and 7.5% dextran 500 000 supplemented with 0.15 M NaCl and the affinity ligand.

It was possible to recover 85% $CD34^+$ cells from the mixture of KG-1a and L929 cells using "free" anti-CD34 as affinity ligands. It was also observed that the same phenomena holded for both types of ligands (anti-CD34 and anti-CD34-poly(NIPAM)), that is, the lower purification was obtained when KG-1a cells were applied to the ATPSs at higher ratios. This may indicate that the amount of anti-CD34, in both "free" and conjugated form, was not enough for the entire separation of a greater number of cells. The decreasing PF with increasing ratio of L929 present in the cell mixture, noted for both types of ligands, may lead us to assumption that there are more separation steps needed in order to obtain better results.

Finally, ATPS composed of 5.6% PEG 8 000 and 7.5% dextran 500 000 supplemented with 0.15 M NaCl and affinity ligand was successfully used for the specific partitioning and recovery of $CD34^+$ stem/progenitor cells from UCB. It was possible to achieve purification factors up to 241 with only one step of partitioning experiment. Considering the accomplished results it seems that ATPS can be faced as an advantageous alternative to traditional techniques for UCB processing like classical ficoll density gradient centrifugation and magnetic sorting.

The data suggest that this novel selection method can be useful for the development of stem cell engineering, providing easy to implement and cost-effective method for purification of target populations.

Several studies, however, are left as ideas for future work. The condition of HSCs after the partitioning in ATPS still remains unclear and it is necessary to examine the morphology of recovered cells. It would be interesting to perform an additional application of recycled Ab-poly(NIPAM) conjugates to the ATPSs, since the successful recycling of such costly ligands seems to be a remarkable approach for the preparative scale. Also, the conjugation of macroaffinity ligand with anti-CD38 mAbs could be performed, in order to remove the more mature cells from the $CD34^+$ population, previously enriched in affinity ATPS. Partitioning studies of "free" anti-CD38 in ATPSs consists of PEG and dextran under different conditions could also give information about feasibility of using anti-CD38 in a "free" form. Moreover, studying the ex-vivo expansion of ATPS-enriched HSCs in the bioreactor system, under dynamic and highly controlled conditions, would allow to verify if cells are still maintaining the primitive $CD34^+CD38^-$ phenotype. Finally, fundaments of affinity ATPSs could be possibly used for the purification of different stem cell populations, leading ultimately to a broad area of applications of affinity ATPSs.

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