PREPARATION AND APPLICATION OF MONOSIZED MAGNETIC PARTICLES IN SELECTIVE CELL SEPARATION

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Some of the basic principles of formation of monosized macroporous particles by the method of "activated swelling" and the preparation of superparamagnetic particles based upon these particles are discussed. A short review of the applications of monodisperse, magnetic polymer particles in cell separation, with emphasis on recent work, is given. Some new work concerning non-specific adhesion of antibodies and cells on different beads, and the prevention of this unwanted phenomenon by help of casein are described. A method for the removal of excess magnetic beads by gradient centrifugation is presented. Positive cell separation, with easy liberation of free cells after magnetic isolation of rosetted cells, is obtained by use of particles with covalently coupled aminophenyl boronic acid at the surface.

1. INTRODUCTION

The idea of using magnetic separation techniques in cell separation is not new, but it has enjoyed a resurgence of interest over the last decade. This has primarily been brought about by the development of new magnetic particles with improved properties for various cell separation procedures.

Immunomagnetic separation (IMS) has several advantages. It permits the target material to be isolated directly from crude solutions such as blood, bone marrow and tissue homogenate. Magnetic solid phase handling of biochemical reactants also simplifies procedures such as change of buffer conditions and repeated washing steps. The extreme monodispersity with respect to both particle size and content of magnetic material characteristic of the Dynabeads (Dynal A/S, Oslo, Norway) assures reproducible and controlled procedures for different cell separation processes.

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Since our own experience with IMS is based solely on Dynabeads, the present discussion focuses on these particles. Reviews on IMS of cells with Dynabeads, covering papers up to 1993, have been presented earlier.[1–3] A comprehensive review on the use of Dynabeads in microbiology, covering papers up to 1994, was given by Olsvik et al.[4] During the last three years about 500 additional papers have appeared, in which the use of Dynabeads in cell separation has played a vital role. These papers have been reviewed by Ugelstad et al.[5] in a paper still in press. The present paper focuses more on the basic principles for preparation of monosized particles, including new types of beads.

2. MONODISPERSE POLYMER PARTICLES

The monosized, magnetizable polymer particles discussed in the present paper, marketed as Dynabeads (Dynal, A/S Oslo, Norway), are prepared from monosized macroporous particles which are magnetized by an in situ formation of ferrimagnetic material inside the pores. The reproducibility in the preparation, as well as the high flexibility in choice of properties such as morphology, surface properties and superparamagnetism of the final particles, can be ascribed to the unique process for preparation of monodisperse macroporous particles by the "activated swelling method" developed by Ugelstad et al.[1,6,7] It therefore seems relevant to review some of the basic features of the preparation of the non-magnetic monodisperse particles in order to understand the unique properties of the Dynabeads, as well as possible improvements, modifications and limitations.

Methods for preparing monosized polymer particles of size up to 0.5 μm in diameter by aqueous emulsion polymerization under strictly controlled conditions have been known for a long time[8]. Due to the low swelling capacity of pure polymer particles (2–5 times by volume), a direct use of such particles as seed for production of "large" monodisperse particles would require that a cycle of swelling and polymerization was repeated several times. Moreover, a process with repeated swelling and polymerization is unsuitable for the preparation of crosslinked and macroporous particles. However, such small particles were found to be very suitable as starting seed for "activated swelling".

The main feature of the process for preparation of large monodisperse particles is the initial "activation" of the small, monodisperse polymer seed particles. In aqueous dispersion, these activated particles are capable of absorbing vinyl monomers and other slightly water-soluble compounds (here denoted Z compounds) in an amount which far exceeds that of the pure polymer particles.

Activation is carried out by preparing monodisperse seed particles which partly or exclusively consist of highly water-insoluble, low molecular weight substances, in the following denoted Y-compounds. Three different processes have been applied to produce activated seeds:

1. Use of chain transfer agents. Small monodisperse seed particles are swollen with monomer and an oil-soluble chain transfer agent, so that the subsequent polymerization gives oligomers of the desired molecular weight. This oligomer functions as a Y-compound.
2. Use of a high ratio of rate of radical formation to rate of polymerization. In this case the seed particles are swollen with monomer and an oil-soluble initiator in a ratio which gives the appropriate low molecular weight of the oligomer formed by the subsequent polymerization.

3. The two-step swelling method, which implies that small monodisperse seed particles are first swollen with a suitable Y-compound, and then in a second step swollen with monomer (Z), followed by polymerization.

As the Y-compound by definition is highly water-insoluble, it is essential that it is added as finely dispersed droplets when used in method 3. This increases the interfacial area of the droplets and thereby the partial molar free energy of the pure Y-compound, and results in an increase both in the rate and the degree of swelling of the seed. In some cases it may be necessary to add some water-soluble organic liquid like ethyl alcohol to the aqueous phase in order to increase the solubility, and thereby the rate of transport of Y. The additive is removed before addition of Z in the second step. If the Z-compound has a relatively low solubility in water, it may be advantageous to add even these compounds as an aqueous emulsion. For the two-step swelling method to function satisfactorily, it is essential that all of the Y-compound has been absorbed into the particles prior to the second step, that is, swelling with the Z-compound. The solubility of Y in the continuous phase is much lower than that of Z, so that no transport of Y out of the particles takes place during swelling with the Z compound.

The equilibrium (or semi-equilibrium) distribution of every component in the various phases present may be calculated from the free energies of mixing. Using the activity of pure component Z_i in a plane (bulk) phase as the reference state, the partial molar free energy of mixing of component "i" in a dispersed phase consisting of *n* components may be written:[7]

$$\Delta\overline{G}_i/RT = \ln\phi_i + \sum_{\substack{j=1\\ j\neq i}}^{n}(1-m_{ij})\phi_j + \sum_{\substack{j=1\\ j\neq i}}^{n}\chi_{ij}\phi_j^2 + \sum_{\substack{j=1\\ j\neq i}}^{n-1}\sum_{\substack{k=j+1\\ k\neq i}}^{n}\phi_j\phi_k(\chi_{ij}+\chi_{ik}-\chi_{jk}m_{ij}) + 2\gamma\overline{V}_i/rRT \quad (1)$$

where ϕ denotes volume fractions, $\overline{V}_i$ is the partial molar volume of "i", $m_{ij} = \overline{V}_i/\overline{V}_j$, χ_{ij} is the interaction parameter per molecule of compound "i" with compound "j", γ is the interfacial tension and r is the equilibrium radius of the swollen particles. For any component ("i") which may be transported between the various phases, seed particles ("a"), dispersed droplets ("b") and continuous phase ("c"), the following condition must be fulfilled at equilibrium:

$$\Delta\overline{G}_{ia} = \Delta\overline{G}_{ib} = \Delta\overline{G}_{ic} \quad (2)$$

By means of Equations (1) and (2), the swelling capacity of various types of seed particles may be calculated. These equations have formed the basis for the development of practical recipes for industrial production of monosized polymer particles, as well as for evaluating model experiments on laboratory scale. A thorough discussion of the application of these equations and the appropriate modifications that should be applied in special cases has been given by Ugelstad et al.[7]

The equation describing the activation of the polymer seed particles with a Y-compound (phase "b"), which is the first step in the two-step swelling method, may be written:

$$\Delta\overline{G}_{Ya}/RT - \Delta\overline{G}_{Yb}/RT = \ln\phi_{Ya} + \phi_{Pa} + \phi_{Pa}^2\chi_{YP} + 2\overline{V}_Y(\gamma_a/r_a - \gamma_b/r_b)/RT = 0 \quad (3)$$

where r_a and r_b are the radii at equilibrium and γ_a and γ_b are the respective interfacial tensions.

For the purpose of describing the further swelling of the activated (polymer/oligomer) particles with a compound Z_1, we may consider a simplified situation where both polymer (P) and Y are completely water-insoluble and present only in the seed particles. Then Z_1 (phase "b") is the only component that may be transported through the aqueous phase. Assuming that the content of water in the organic phases may be neglected, and that the interaction parameters are independent of concentration, the equilibrium swelling of the activated seed particles may be calculated from the equation:

$$\Delta\overline{G}_{1a}/RT - \Delta\overline{G}_{1b}/RT = \ln\phi_{1a} + (1-m_{1P})\phi_{Pa} + (1-m_{1Y})\phi_{Ya} + \phi_{Pa}^2\chi_{1P} + \phi_{Ya}^2\chi_{1Y} + \phi_{Ya}\phi_{Pa}(\chi_{1Y}+\chi_{1P}-\chi_{YP}m_{1Y}) + 2\overline{V}_1(\gamma_a/r_a-\gamma_b/r_b)/RT = 0 \quad (4)$$

where index "1" refers to Z_1.

Although Equation (4) is based on some simplifying assumptions, it is well suited to illustrate the main point of the principle of "activated swelling", namely that the presence of a low molecular weight, water-insoluble compound (Y) in the seed particles may bring the particles to absorb orders of magnitude more of low molecular weight, slightly water-soluble compounds, like vinyl monomers, than is possible by swelling of particles of pure polymer. This increase in swelling capacity becomes more pronounced the higher the initial radius (r_{ao}) of the activated seed particles and the smaller the droplets of the monomer.

Figure 1 shows the swelling of a polymer/oligomer particle, expressed as volume (V_1) of Z_1 absorbed per unit volume of (V_p + V_Y), versus γ_a/r_{ao}. The curves were calculated using Equation (4), with $V_Y+V_P = 1$, $m_{1Y} = 0.2$, $\chi_{1Y} = \chi_{1P} = 0.5$, $\chi_{YP} = 0$, $\overline{V}_1 = 10^{-4}$ m^3/mol and T = 323 K.

From Equation (4) it is seen that the degree of swelling may be increased by reducing the radius of the b-droplets. In fact, the equation predicts that it should in principle be

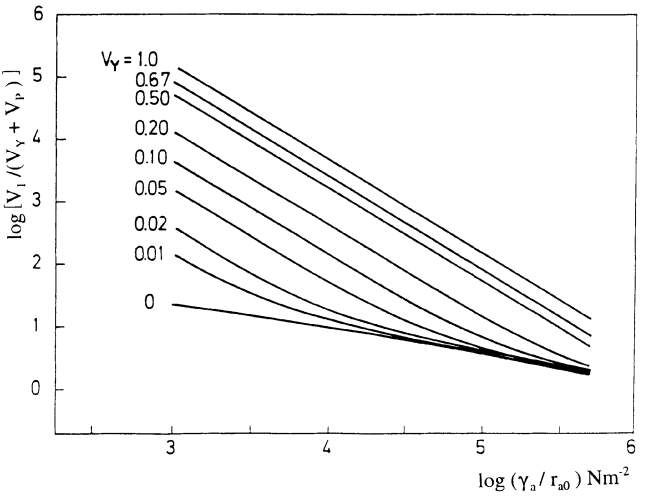

Figure 1. Swelling capacity of activated particles versus γ_a/r_{ao} for different values of V_Y.

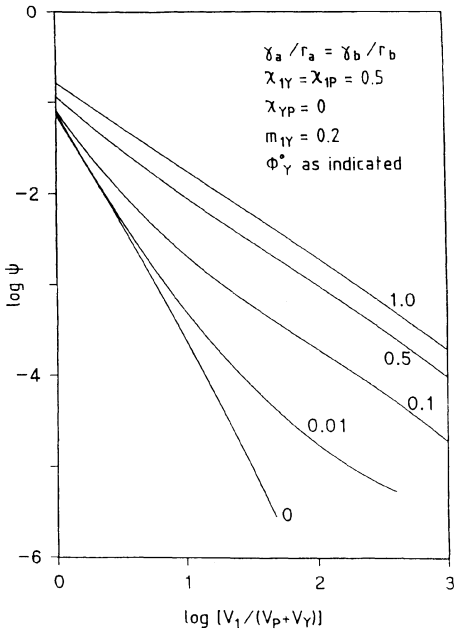

Figure 2. Swelling of polymer-oligomer particles with Z_1. Driving chemical potential (ψ) versus swelling ratio for various initial volume fractions of Y.

possible to obtain infinite swelling when $r_b < r_a$. In practice this is very difficult to realize because r_b increases with time due to interdroplet diffusion.

It may be instructive to consider what may be called the "driving chemical potential" (ψ) for the swelling process. In reality, ψ illustrates the advantages of the two-step method without including the effect of the radii, that is, the surface energy terms. Defining ψ by:

$$\psi = -(\Delta \overline{G}^{p}_{1a} - \Delta \overline{G}^{p}_{1b}) / RT \qquad (5)$$

where index 'p' refers to a plane surface ($r = \infty$), the effect of the degree of swelling on the driving chemical potential may be illustrated in a log-log diagram. Figure 2 shows how ψ varies with the degree of swelling, $V_1/(V_Y+V_P)$, of a polymer particle with different initial volume fractions (ϕ_Y^0) of a component Y having the same value of m_{1Y} and the same interaction parameters as those in Figure 1.

It appears that as little as 10% of Y in the particles at start gives a noticeable increase in driving free energy of swelling compared to that of pure polymer particles ($\phi_Y^0 = 0$). The increase becomes markedly more pronounced as the swelling ratio increases. Thus, Figure 2 in an unambiguous way illustrates the tremendous advantage of the method of activated swelling. Examples of Y-compounds that have been used are hexadecane, dioctyl adipate and dioctanoyl peroxide. The latter may play a dual role, both as Y-compound promoting swelling and as initiator for the subsequent polymerization. Normally, the particles resulting from the first swelling step contain from 50 to 90 % by volume of

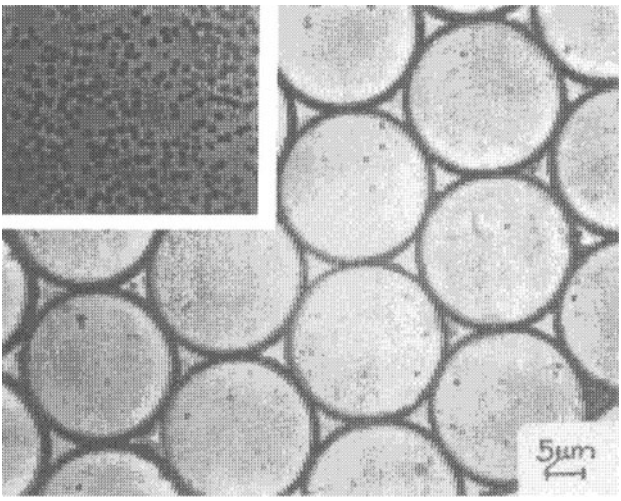

Figure 3. Monodisperse particles swollen with toluene. Insert: 1 µm dioctyladipate activated polystyrene seed.

an oligomeric substance, and are therefore able to absorb from 10 to 1000 times more of slightly water-soluble Z-compounds than pure polymer particles.

The tremendous increase in swelling capacity predicted by Equation (4) is demonstrated in Figure 3 which shows dioctyl adipate (Y) activated, 1 µm polystyrene particles swollen about 8000 times with toluene. Referring to Equation (4), the relatively low molecular weight of dioctyl adipate (Y) is responsible for the high entropy of swelling which results from replacing a part of the polymer in a pure polymer particle with Y.

A very interesting consequence of the importance of the entropy term, $(1\text{-}m_{1Y})\,\phi_{Ya}$, which was also verified experimentally, is that the swelling most often is determined solely by the amount of Y in the particles, so that also crosslinked particles may "swell" with Z-compounds to approximately the same degree as non-crosslinked particles. It appears that the swelling takes place in a layer of Y which is formed outside the inner polymer core. The low viscosity in this layer may account for the fact that the rate of "swelling" with Z is faster with crosslinked particles. The degree of swelling is approximately the same as with non-crosslinked particles and may be calculated from Equation (4) by omitting the terms with the index Pa. The use of crosslinked seed particles for the production of large monodisperse particles completely eliminates the effect of the seed polymer on the morphology of the final particles.

As shown by Ugelstad et al.,[7] the seed may in some cases be completely expelled from the particle during the final polymerization. Figure 4 shows 4.2 µm poly(methyl methacrylate (73%) hydroxyethyl methacrylate (20%) ethylene glycol dimethacrylate (7%)) particles, from which the polystyrene seeds have been expelled during polymerization, thus creating a void corresponding to the size of the original seed particle. This is most likely to happen if the seed polymer and the polymer formed from the monomer added in the second step are completely immiscible, and moreover that the monomer added in the second step is far in excess of the polymer in the seed, as this will provide a lasting low viscosity and ease transport of the phase-separated seed polymer. A complete removal of the seed may be of interest in systems where the seed polymer impart unwanted properties to the final particles.

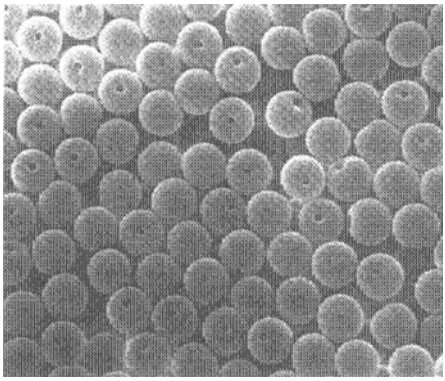

Figure 4. SEM illustrating the expulsion of 1 μm polystyrene seed particles.

3. MONOSIZED MACROPOROUS PARTICLES

The characteristic feature of the method of activated swelling when used for the production of monodisperse macroporous particles is, in the first step, the preparation of droplets which contain all the ingredients necessary for the production of the final particles, i.e. the monomer mixture including the crosslinking components and the inert liquids (porogens) which after polymerization are removed to obtain the macroporous structure. The porogen should therefore also be a Z compound in the way that it is slightly soluble in water. After polymerization, the porogen is removed by extraction, giving macroporous polymer particles. Preferentially, oil-soluble initiators which may be introduced into the particles together with, or before the addition of the Z compounds, should be used.

Another most important feature of the method of activated swelling for production of macroporous particles is that the original polymer seed makes up only a very small fraction (less than 1%) of the final particle. The presence of polymer is undesirable for several reasons. It is difficult to remove the polymer by extraction after polymerization. Polymer, even in small amounts, tends to create a fraction of large pores, independent of what other porogen is applied. It also tends to give formation of a skin on the surface of the particles. By preparation of small particles, a too high proportion of seed polymer to added Z-compounds leads to the formation of a rough, dented surface. A very low content of seed polymer allows formation of particles with a smooth surface, even for relatively small particles. Likewise, it provides a high degree of freedom in choice of monomers and porogens, and thereby a good control of pore size and pore size distribution. The use of crosslinked seed particles may provide methods to totally eliminate the effect of polymer on the pore size distribution of the macroporous particles.

The macroporous particles which have been used for the preparation of magnetic particles have for the most been based upon styrene-divinyl benzene. In that case, the general experience is that use of a porogen with a solubility parameter close to the one of polystyrene (δ = 17.6 $(J/cm^3)^{1/2}$) will give small pores, while a polar porogen with a solubility parameter considerably higher or a highly nonpolar porogen with a considerably lower solubility parameter than this value will tend to give large pores. Put in another way, porogens with a high solvency or swelling capacity for the polymer formed results in small pores, while a low solvency favor the formation of large pores. The fact that poly-

mer, when present, always result in the formation of a fraction of large pores, is understandable in view of the general trend to mutual non-solubility of different polymers.

4. MAGNETIZATION OF MACROPOROUS POLYMER PARTICLES

The procedures for preparing magnetic polymer particles from the monosized macroporous particles start out by introducing $-NO_2$ or $-ONO_2$ groups covalently coupled to the surface inside the pores of the particles. This treatment also renders the particles hydrophilic. Therefore, when these particles are dispersed in an aqueous solution of Fe^{2+} salts, the liquid immediately fills up the pores. By proper choice of reaction conditions, Fe^{2+} reacts with $-NO_2$ or $-ONO_2$ groups to iron hydroxides which precipitate inside the pores. This creates a concentration potential resulting in a continuous diffusion of iron salts into the pores until the external phase is completely depleted of iron salts. Upon heating, particles having magnetic iron oxides deposited as small grains evenly distributed through the pore volume of the particle are obtained.

The magnetization process may be simplified as:

$$-NO_2 + 6\ Fe(OH)_2 \Rightarrow -NH_2 + 3\ \gamma Fe_2O_3 + 5\ H_2O$$

After magnetization, the particles are covered with a polymer which is intended to fill up the pores and also may provide surface groups which may be used for binding of proteins, nucleotides etc. The $-NH_2$ groups formed on the pore surface provide a versatile, reactive group for both addition and condensation polymerization, as well as for reactions with vinyl compounds, thus making it easy to fill the pores with polymer.

The composition of the iron deposits in the polymer particles has been analyzed by Mössbauer spectroscopy[9] and X-ray powder diffraction measurements.[10] According to both analysis, the iron is mainly present as γ-Fe_2O_3 (maghemite) which may be considered as a fully oxidized magnetite. Magnetite (Fe_3O_4) is an inverse spinel while maghemite is a deficient spinel.[11–13] Maghemite preserves the same cubic structure as magnetite, achieved through ejection of 11% of Fe from the magnetite structure, thereby creating vacancies. All the magnetic ions in γ-Fe_2O_3 are identical, namely Fe^{3+}, and ferrimagnetism arises from an unequal distribution of these ions in the tetrahedral (A)-sites and octahedral (B)-sites of the unit cell. In ferrites with spinel structure, the magnetic momentums resulting from the spin of the 3d electrons of the ions are expected to be oriented in opposite direction for the (A) and (B) sited ions. The resulting magnetism is then the difference between the magnetization of the octahedral lattice (B) and the tetrahedral lattice (A). Maghemite is almost as magnetic as magnetite, the saturation magnetizations at 20°C being 76 and 92 emu/g respectively.

Figure 5 shows a typical magnetization curve of M-450 particles at room temperature. The measurements were carried out with a vibrating sample magnetometer (VSM, Princeton Applied Research) using a Ni probe as standard.[11] The magnetization curve shows no hysteresis, accordingly both retentivity and coercivity are zero.

This behavior can be explained in terms of superparamagnetism.[11,12] The small grains of magnetic oxide within the polymer carrier behave as single domains with a magnetic dipole moment proportional to their volume. In the case of geometrically fixed magnetic particles, thermal activation is the most probable mechanism to reach thermodynamic equilibrium. If single domain particles become small enough, energy fluctuations could overcome the anisotropy forces and spontaneously reverse the magnetiza-

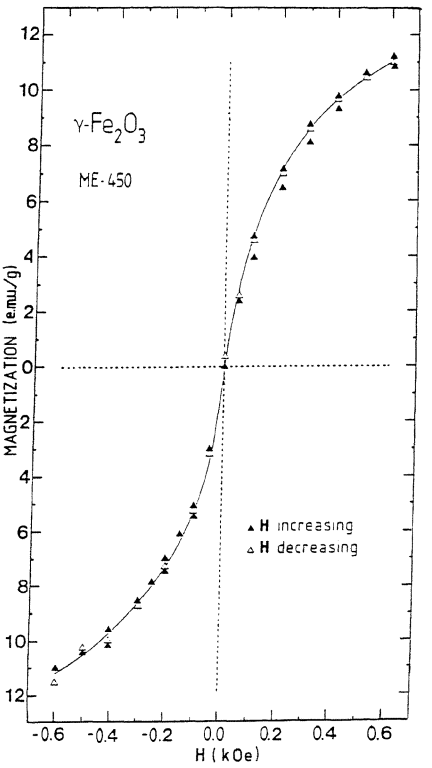

Figure 5. Magnetization curve for 4.5 μm magnetic particles (M-450) at room temperature.

tion of a particle from one direction to the other. The approach to thermal equilibrium is governed by a relaxation time constant of the generalized form:

$$1/t = f_o \exp(-\Delta E/kT)$$

where t is the relaxation time, f_o is the frequency factor ~10^9 sec^{-1}), ΔE is an energy barrier opposing spontaneous reversal.

As the anisotropic energy ΔE is proportional to the volume V_p of a magnetic grain, we have:

$$1/t = f_o \exp(-K \cdot V_p/kT)$$

where K is a proportionality constant. It is evident that the grain size has a tremendous effect on the relaxation time. For practical purposes, a material is said to be superparamagnetic when the relaxation time (t) is less than 100 seconds. Using this value gives a critical size for uniaxial magnetic particles:

$$V_{p,crit} = 25kT / K$$

Estimates of critical values for V_p vary a lot in the literature. For magnetite and maghemite grains, diameters as high as 20–40 nm are given. In commercial superparamagnetic ferrofluids consisting of magnetite, the particle size is normally below 10 nm.

For practical reasons, the magnetic properties of magnetic polymer particles are characterized by measuring their magnetic susceptibility. These measurements are usually carried out using the Faraday method[11] which is based on measurement of the force F_x acting on a body when it is placed in a nonuniform magnetic field. F_x may be expressed by:

$$F_x = \kappa \cdot m \cdot H \cdot dH/dx$$

where κ is the magnetic susceptibility, m is sample mass, H is the magnetic field strength and dH/dx is the field gradient in the force direction. From calibrations with linear standards of known susceptibility such as solid $HgCo(NCS)_4$,[14] the product $H \cdot dH/dx$ can be determined. It is stressed that the magnetic susceptibility of ferro- and ferrimagnetic materials is a function of the field strength. The magnetization of these materials will level out and reach saturation magnetization with increasing magnetic field strength. Therefore, any comparison, as well as control measurements, have to be carried out at fixed conditions.

The superparamagnetic property of magnetic polymer particles is of course very important from a practical point of view as it means that suspended particles may repeatedly be collected by a magnet, and immediately redispersed once the magnetic field is removed.

The standard deviation of the magnetic susceptibility of individual Dynabead M-450 particles is less than 5%.[15]

Some of the most common types of Dynabeads are shown in Figure 6. M-450 (a) has a hydrophobic epoxy resin surface, and may be used without further treatment for binding of ligands. M-280 (b) contains hydrophobic urethan resin with short-chain poly(ethylene glycol) groups $-(CH_2-CH_2O)_{2-4}H$ on the surface. M-270 (c) is a newly developed particle which, like M-450, has a relatively smooth surface, but is more hydrophilic. The main properties of the particles are given in Table 1.

Covalent coupling of ligands to Dynabeads may take place by direct coupling to groups originating from the polymer, as may be the case with particles containing epoxy resins, or by activation of -OH, -COOH or $-NH_2$ groups at the surface. Chemical coupling

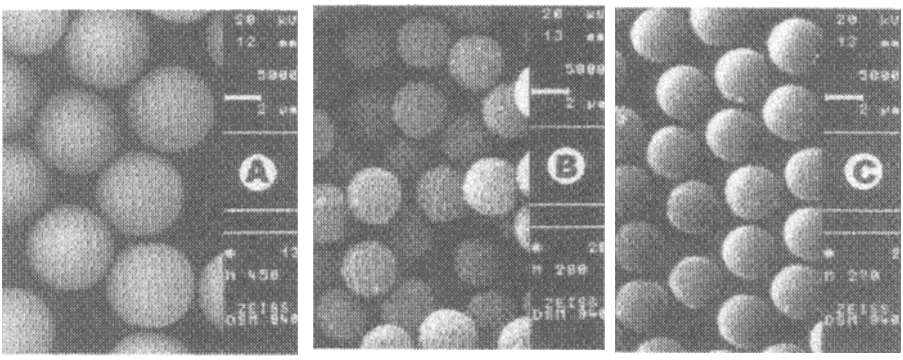

Figure 6. SEM pictures of various Dynabeads.

Table 1. Properties of M-450, M-280 and M-270 Dynabeads (average values)

Particle type	Diameter μm	Surface area m^2/g	Fe content weight %	Density g/cm^3	Particles per mg
M-450	4.5	3-5	22	1.5	$1.5 \cdot 10^7$
M-280	2.8	4-6	17	1.3	$7 \cdot 10^7$
M-270	2.8	3-5	20	1.6	$5 \cdot 10^7$

of ligands, as for instance antibodies, to the hydrophobic M-450 and M-280 beads, are preceded by a rapid adsorption of the ligand. This provides the high local surface concentration required to obtain an efficient covalent coupling of the ligand. With strongly hydrophilic surfaces, achieving a good immobilization yield may be a problem. Both yield and rate may be increased by using large concentrations of an inorganic salt, like ammonium sulfate, or by adding the antibody in form of a microemulsion.[16]

5. APPLICATIONS OF MAGNETIC BEADS

Some references to recent papers where Dynabeads have played a vital role in selective cell separations of both eukaryotic and prokaryotic cells are collected in Table 2. Although the table contains only a few of the vast amount of papers published, it illustrates the versatility of immunomagnetic separation (IMS) with Dynabeads in cell separation procedures. Most often, IMS of eukaryotic cells is carried out on peripheral blood (PB) or bone marrow (BM), either directly or after density gradient centrifugation, to obtain mononuclear cells (PBMC, BMMC). Dynabeads have been increasingly used for IMS of cells from various tissues.

The use of Dynabeads for the isolation of bacteria and viruses was a natural following up of IMS on eukaryotic cells, and has grown to become a very important field of application. Current uses includes the isolation and analysis of different bacteria and viruses from clinical samples, and monitoring of bacterial or other pathogenic contamination, for example in food or waste water.

In addition, a few references to molecular biology, immunoprecipitation and immunoassays where these beads are used, are given in Table 3.

Some detailed examples cited in Table 2, in which IMS with Dynabeads plays an essential role, both clinically and for functional studies of selected subfractions of cells, will be covered in section 5.2.

5.1. Experimental Procedures for IMS

IMS of cells implies the use of a bead-antibody system causing the bead to be selectively attached to target cells when added to a cell suspension. After incubation, cells with attached beads and excess beads are isolated with the help of a magnet. Most often, a monoclonal antibody (mAb) is used in combination with IMS due to its specificity towards the target cell. The various antibody application methods in IMS of cells may be classified as follows:

5.1.1. Direct Method. The mAb is coupled to the beads, which are then added directly to the sample. Ideally, the mAb should be oriented with its Fc part towards the bead

Table 2. Some recent applications of magnetic beads (Dynabeads) in cell separation. DM = direct method, IM = indirect method, BM = bone marrow, PBMC = peripheral blood mononuclear cells, PBPC = peripheral blood progenitor cells

Applications	Keywords	References
Eukaryotic cells		
Purging of cancer cells	BM/Leukapheresis prod.,T-,B-, breast cancer, hepatoma cells	17,18,19
	Mobilized PBPC, B-cells, IM	20
	BM, T-cells, IM	21
Isolation/functionality of cancer cells	Breast cancer cells, negative/positive selection, DM	22
	Blast cells, negative selection, effect of interleukin 10	23
T_8-depletion in allogenic transplantation	BM, DM	24
	BM, IM	25
IMS in the studies of HIV and AIDS	PBMC, isolation of $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$ cells	26,27,28,29
	PBMC, negative selection of $CD8^+$cells, IM	30
	PBMC, isolation of $CD4^+CD7^-$ cells	31
Isolation of NK cells	PBMC, Positive selection, IM	32,33
Isolation of granulocytes (from blood)	Eosinophils, negative selection, DM	34
	Eosinophils, negative selection, IM	35, 36
	Basophils, positive selection, IM	37
Isolation of cells from various tissues	Review (leukocytes and non-leukocytes)	38
	Human skin dendritic cells, negative selection, IM	39
	Thymic epithelial cells, IM	40
	Endothelial cells, positive selection, DM	41,42
	Mast cells, positive selection, IM	43
IMS of progenitor stem cells/ clinical aspects	Leukapheresis prod., Baxter Isolex 300	18
IMS of progenitor stem cells/ functional studies	Leukapheresis prod., positive selection, IM	44
	BM, positive selection, DM	45
	BM, positive selection, IM	46
	BM, negative selection followed by positive selection	47
	BM and leukapheresis prod., positive selection	48
	PBMC, positive selection, DM	49
	PBPC, Baxter Isolex 50	50
	BM, positive selection, DM	51
Isolation of cell compartments	Golgi fractions	52
	Endosomal vesicles, Golgi complex	53
Prokaryotic cells		
Isolation/identification of bacteria, viruses and parasites	Reviews	4,54
	Isolation of *Helicobacter pylori*	55
	Isolation of *E. coli*	56,57
	Isolation of *Salmonella*	58,59,60

so that the Fab region is pointing outwards from the bead. When using IgG mAbs, the system may in some cases suffer from steric hindrance.

5.1.2. Two-Step Direct Method. A secondary antibody is attached to the bead in a first step. This antibody (e.g. sheep anti mouse IgG, SAM) may be directed towards the Fc part of the primary mouse mAb added in the next step. The secondary antibody acts as a spacer arm and helps to orient the primary antibody. The much larger IgM molecule (pentamer) is most often coupled directly to the bead.

Table 3. Some recent applications of magnetic beads (Dynabeads) in molecular biology, immunoprecipitation and immunoassays

Applications	Keywords	References
Molecular biology		
Isolation/identification of DNA and RNA	Review	61
Immunoprecipitation		
Isolation of various proteins	Lipopolysaccaride-binding protein from granulocytes	62
	Human transferrin receptor	63
	Major histocompatibility complex I and II	64
Immunoassays		
Selection/detection of various proteins	Review	65
	Tumor necrosis factor	66
	Antibodies against alloantigens on human platelet glycoproteins	67
	In vivo capture of proteins from body fluids	68

5.1.3. Indirect Method. In a first step, the cell suspension is incubated with the primary mAb, e.g. mouse mAb, or a cocktail of mouse mAbs, which bind to the target cells. After incubation, the excess of antibodies is removed by washing. Thereafter, the magnetic beads, which contain secondary sheep anti-mouse antibody on the surface, are added, permitting the beads to bind rapidly and firmly to the primary antibodies on the target cells.

With IMS of eukaryotic cells, one differentiates between negative and positive selection of cells.

5.1.4. Negative Selection. Unwanted cells are rosetted with magnetic beads, removed by help of a magnet and discharged. Purging of bone marrow for tumor cells used in combination with high dose therapy treatment of cancer patients represents an example of negative selection of cells. Negative selection may also involve preparation of a specific subset of cells by immunomagnetic removal of all other cells in the suspension. Both the direct and the indirect method are applied for negative selection. The negative selection of cells has the advantage that the purification process does not involve any direct contact with the target cells. This may be particularly important if the mAb used to identify the target cells reacts with membrane structures that influence cell functionality. Negative selection has its limitations. If the subsets of cells to be selected are present in a low concentration, negative selection may give low yield and purity due to non-specific loss of the cell to be isolated, or to an insufficient removal of unwanted cells.

5.1.5. Positive Cell Selection. By use of IMS, the target cells are isolated from the cell suspension. The bead/cell complexes separated can be further characterized directly, or the bead may be detached from the target cells after isolation to give free cells. Detachment of Dynabeads from isolated target cells may be accomplished in different ways:

a. By placing the rosetted cells in culture at 37 °C.
b. By enzymatic splitting of the antigens with Chymopapain.[69]
c. By enzymatic splitting of antibodies coupled via a DNA linker.[70]
d. By incubation with DETACHaBEAD, an anti-Fab fragment that bind to the Fab part of the primary mAb and thereby reverses the binding between the monoclonal antibody on the bead and the antigen on the cell.[71]

e. By a synthetic peptide which binds specifically to the antigen binding site and competes with the antibody bead complexes (Baxter). Both system d and e give detached cells with unchanged antigen expression, and no mAbs are left on the surface.

f. By use of Dynabeads with $-B(OH)_3$ groups at the surface, allowing attachment of antibodies through formation of a complex between the $-B(OH)_3$ groups and the carbohydrate units on the Fc part of the antibodies (discussed in 5.2.5.).

Cell surface molecules on different leukocytes to which monoclonal antibodies have been developed, are identified by the internationally recognized nomenclature of cluster differentiation antigens (CD). When discussing magnetic beads coated with a particular monoclonal antibody towards one of the antigens, this is often shortened to for example Dynabead CD4, or more specifically M-450 CD4. Precoated and commercially available Dynabeads for direct IMS of human cells include monoclonal antibodies directed towards different subsets of human blood cells, T-lymphocytes (CD2, CD3, CD4, CD8), B-lymphocytes (CD19), monocytes (CD14), granulocytes (CD15), stem cells (CD34), leukocytes (CD45), erythroblasts (CD71), and HLA class II cells. Beads for separation of mouse blood cells have also been prepared. In addition, there is a Dynabead anti-epithelial cell for enrichment of carcinoma cells from blood and bone marrow. Dynabeads coated with different antibodies such as Sheep anti-Mouse (SAM) IgG and IgG1, Goat anti-Mouse IgG, Rat anti-Mouse IgG1, IgG2a and IgG2b, Rat anti-Mouse IgM and Sheep anti-Rat IgG, are also available. Dynabeads M-280 coated with streptavidin on the surface are available, and can be used for immobilization of biotinylated antibodies, lectins and DNA. Detailed descriptions of the various procedures applied for effective cell separation by IMS with Dynabeads are provided by the distributor of the Dynabeads (DYNAL A.S., Oslo, Norway). For additional information, a detailed description with experimental protocols for various beads and cells have recently been given.[72] Important factors that should be considered in order to obtain efficient cell separation are the bead to cell ratio and the total concentration of beads. According to our experience, this ratio should preferably be > 5 in most cases. However, independent of the concentration of target cells, there is a lower limit in the concentration of beads to be applied in order to obtain an effective and sufficiently rapid attachment of the beads to the target cells. For M-450 and M-280, the optimal concentration of beads is 10^7-5×10^7 particles per ml. Therefore, when working with very low concentration of target cells, a very high ratio of number of beads to number of target cells are used.

5.2. IMS of eukaryotic Cells

5.2.1. Purging of Cancer Cells. The use of monosized magnetic beads for selective separation of cells was initiated by Kemshead, Rembaum and Ugelstad in 1979. They used the beads for removal of neurolbasts from bone marrow in connection with autologous bone marrow transplantation. This technique has later been extended to other tumors, and has been modified with regard to both the type of beads and the purging procedure. Bone marrow purging in connection with autologous bone marrow transplantation requires a combination of high efficiency in removal of tumor cells and high recovery of hematopoietic stem cells. Most often the indirect method has been used, but attempts have been made to simplify the method. Kvalheim et al.[17,18,73] attached the anti-B cell IgM mAbs directly to the beads. In model experiments with 10% Burkitt lymphoma cells admixed to bone marrow, purging with a combination of mAbs gave > log 5 depletion. The

method was tested successfully in patients.[74] Recently, it has been reported that the direct immunomagnetic purging procedure can efficiently deplete cancer cells using a mixture of beads with sheep anti-mouse antibody, coated with individual IgG mAbs.[18] The anti-breast cancer antibodies used all had high affinity. In spite of these results, it is agreed upon that the direct method has its limitations because the number of suitable IgMs is limited, and experiments with IgG mAbs directly coupled to the beads give unsatisfactory purging when the mAb affinity is low. The indirect method allows the use of a cocktail of antibodies, which eliminates the risk that tumor cells may escape immunomagnetic purging because of their lack of expression of the antigen recognized by the individual mAbs.[18] Results published so far indicate that the purging efficiency is superior when repeated cycles of purging are done. Only a second treatment with magnetic beads is required, i.e. with beads with covalently attached mAbs, IgG or IgM, in the case of direct purging, or with beads coated with a secondary antibody when applying indirect purging. Analysis of purged marrow using polymerase chain reaction (PCR) techniques verify the excellent results of model experiments with clonogenic determination of remaining tumor cells after purging.[75,76] In addition, IMS has proven to give an insignificant cell loss, with recovery of 70–80% of hematopoietic progenitor cells as determined by CFU-GM (colony forming units of granulocytes and macrophages).[19]

Since 1990, there has been a shift towards the use of mobilized peripheral blood progenitor cells (PBPC) instead of stem cells from bone marrow because of the favorable reconstitution kinetics of hematopoiesis. Although peripheral blood frequently seems to contain fewer tumor cells than bone marrow, it has been shown that contamination of PBPC with tumor cells has to be considered in patients with various cancer cells.[77–79] Therefore IMS purging procedures have also been developed for PBPC.[18]

5.2.2. Detection of Cancer Cells in Blood. The M-280 beads turned out to be so successful for selective isolation of very small cell populations in blood and bone marrow that it came natural to use such beads for the isolation of cancer cells in blood. First of all it is of value for the prognosis to know if there are cancer cells in circulation. Such circulating tumor cells are furthermore important objects for further research on the metastatic process. Different methods for detection of very low concentrations of tumor cells in PBMC have been investigated. Preferentially, the method should be able to detect as few as 1–2 tumor cells in 2×10^6 mononuclear cells (MC). Obviously the direct application of immunocytochemistry on MC samples would lead to preparation of very large number of slides cytospin) due to the restricted number of cells that can be centrifuged down on each slide (5×10^5 cells). An alternative approach would be to enrich for tumor cells by immunomagnetic isolation before visualization of the tumor cells (referred to as IMS-standard). However, one problem is that the cells contain a large number of magnetic beads which makes it difficult to identify the tumor cells by immunocytochemistry. Investigations with PBMC infected with breast cancer cells showed that 3 cytospins almost buried the cancer cells.

Quite recently, we have developed a method which may possibly solve this problem. The method is based upon a gradient centrifugation technique (referred to as IMS-centrifugation) whereby the excess of magnetic beads, having the highest density, is separated from the tumor cells which even when fully rosetted with beads, have a distinct lower density. By this method, which is described in more detail in the Appendix, one may remove >95% of the excess of M-280 beads while maintaining >75% of the target cells. Figure 7 shows rosetted PM1 cells isolated by immunomagnetic separation using M-280 beads coated with 9189 mAb, by the IMS-standard method (A) and the IMS-centrifugation

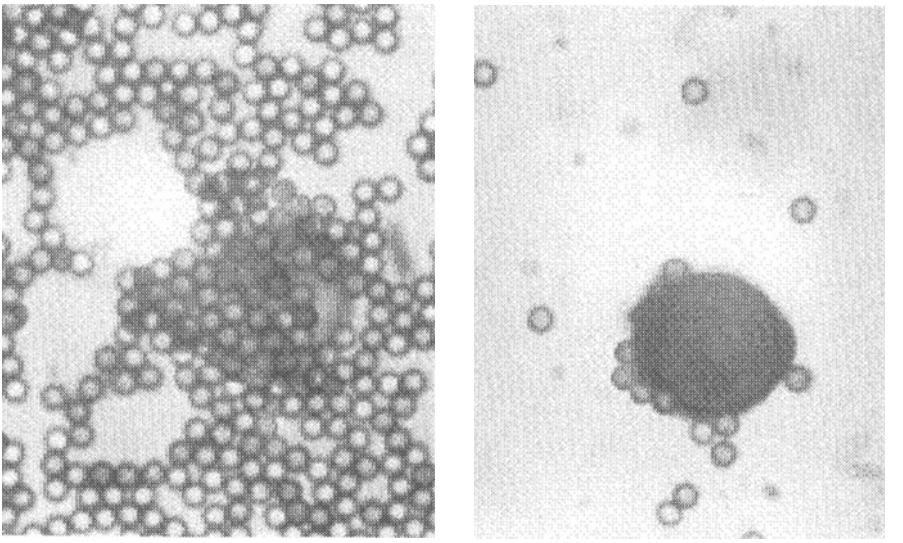

Figure 7. PM1 cells detected by (A) IMS-standard method, and (B) IMS-centrifugation method. The slides were immunocytochemically stained with alkaline-phosphatase-conjugated A45-B/B3 anti-cytokeratin mAB (Fab).

method (B). The tumor cells were visualized by immunocytochemical staining using an anti-cytokeratin Ab, which reacts with all cells containing cytokeratin, i.e. epithelial and mesothelial cells. With the IMS-standard method the rosetted cells were centrifuged down to 3 cytospins, and with the IMS-centrifugation method 2 cytospins were used. The gradient centrifugation method represents a drastic improvement, as it is possible to examine much more cells, still ending up with the same number of cytospins for tumor cell diagnosis as with the IMS-standard method. This method is especially valuable in cases where subsets of cells, which are present in low concentration, have to be isolated.

Quite recently, Naume et al.[22] performed a study where they determined cancer cells in bone marrow and peripheral blood by a negative selection, removing all non-tumor cells by help of M-450 coated with CD45. The success of this method is dependent upon a low non-specific binding of cancer cells to the M-450 CD45 beads, which is present in large excess of the cancer cells.

5.2.3. IMS of Progenitor Stem Cells. An alternative strategy in autologous, as well as in allogenic, transplantation is to reinfuse $CD34^+$ cells isolated with a system of beads and mAbs. In the case that the $CD34^+$ cells have been isolated by positive selection, detachment of the beads include splitting with chymopapain, DETACHaBEAD and the Baxter system. The isolation and liberation of progenitor stem cells has been automated very recently (Baxter Isolex 300). Kvalheim et al.[18] reported clinical experience with $CD34^+$ enrichment from leukapheresis products from patients with breast cancer and non-Hodkin's lymphomas, applying the indirect method. Autografts consisting of isolated $CD34^+$ progenitor cells from PBPC appear to give reconstitution of hematopoiesis after high dose therapy similar to that of unmanipulated PBPC[18]. Since most tumor cells do not express CD34 antigen it might have been anticipated that isolated $CD34^+$ cells would not contain malignant cells. However, only a 2–4 log tumor depletion has been achieved in breast can-

cer patients when $CD34^+$ enrichment was used[80]. Likewise, in lymphoma patients, B cells were found among the isolated cells even when the purity of $CD34^+$ cells reached 99.7%.[18] Isolated $CD34^+$ cells have been subjected to numerous functional studies, and some examples are listed in Table 2.

5.2.4. Non-Specific Binding of Proteins and Cells to the Bead Surface: The Effect of Casein. In some cases, non-specific binding may constitute an obstacle to an effective use of the beads. This is the case in the development of diagnostic test systems in which immunologically active antibodies are coupled to the surface. Any non-specific interaction of complementary antigen and non-specific interaction with an optional second labeled antibody should be prevented. Also, absence of non-specific binding of antibodies at the bead surface is a prerequisite for using the beads with covalently coupled protein A for extraction and subsequent liberation of monoclonal antibodies from ascites. In some cases of positive separation of cells where the cells are liberated from the beads after isolation, the detachment of beads from the cells are brought about by breaking specific bonds between the beads and antibodies. In that case, any non-specific binding of antibodies to the beads may effectively hinder liberation of the cells.

Proteins are rapidly adsorbed on hydrophobic surfaces in what appears to be an irreversible process, as a dilution with pure water does not bring about any desorption of the adsorbed proteins. Protein adsorbed at the surface may undergo time dependent conformational changes to allow formation of additional contacts with the surface. Consequently, several models of protein adsorption include a transition from a reversibly adsorbed state to a more tightly held state, the latter being brought about by molecular restructuring at the surface. An adsorbed protein may be displaced by addition of a solution of another protein with a higher affinity for the bead surface. This detachment is highly dependent on the time and temperature for the first adsorption process, and likewise of time and temperature for the replacement reaction.[81] Elgersma et al.[81] compared the binding of bovine serum albumin (BSA) and a number of monoclonal antibodies to polystyrene surfaces, and they found that preadsorbed IgG was more easily displaced by BSA than the converse. The same authors also demonstrated the effect of time, both for the preadsorption of IgG and the subsequent displacement with BSA.

When comparing different proteins for their capacity in preventing subsequent binding of IgG to hydrophobic particle surfaces, Vogt et al.[82] demonstrated that casein was far more effective at low concentrations than BSA, and suggested that it might be used as an effective blocking agent in ELISAs.

Casein is a mixture of related phosphoproteins occurring in milk and has a distinct amphiphilic character with separate hydrophilic and hydrophobic regions, thus rendering its macromolecular structure well suited to function as an emulsifying agent. The combination of a disorganized structure and substantial hydrophobicity, coupled with a relatively low molecular weight, enables it to spread rapidly and efficiently on hydrophobic surfaces. Thus we found that casein was not only extremely efficient in preventing adsorption of IgG to our hydrophobic magnetic particles. We could also demonstrate that casein was far more effective in effecting a displacement of preadsorbed IgG from hydrophobic particle surfaces than any other protein investigated so far.[83]

With beads having a relatively hydrophobic surface, a preadsorption of the antibody occurs, ensuring a rapid and effective binding of the antibody. The preadsorption of IgG antibody also conveys a favorable orientation of the IgG molecule, with the more hydrophobic Fc part pointing to the surface and the Fab parts pointing out from the beads. For some applications, the hydrophobicity represents a disadvantage with regard to unwanted

non-specific binding of various substances. A number of more hydrophilic beads have been investigated, and recently a new type of beads, M-270, has been brought on the market. These beads have a relatively smooth surface, like M-450, but are far more hydrophilic. To obtain a covalent binding of antibodies to the M-270, it is necessary to precipitate IgG on the surface with an inorganic salt such as $(NH_4)_2SO_4$.

Contamination of non-target cells (e.g. cancer cells) in direct positive cell separation may be a problem in cases were the target cells are present in low concentrations, as for instance in the positive isolation of stem cells. As mentioned in section 5.2.3. only a 2–4 log tumor depletion has been obtained in patients with breast cancer when CD34 enrichment was used.[80] There are reasons to believe that non-specific binding of cancer cells to the beads may contribute to the presence of tumor cells in autografts. It has been demonstrated that tumor cells, even at 4°C, bind to M-450 beads non-specifically to a much higher degree than normal bone marrow cells[84]. We have started a thorough investigation on non-specific binding of various types of cancer cells to various types of beads.[83]

Preliminary results may be summarized as follows: 1) All types of cancer cells show a higher non-specific binding than normal PBMC. 2) Non-specific binding varies considerably between different cancer cells. 3) On hydrophobic surfaces, casein is vastly superior to BSA in preventing non-specific binding. 4) On hydrophilic surfaces, the non-specific binding is as expected considerably lower, but even there the effect of casein is significant.

Casein also had no effect on the antibodies covalently attached to the beads before casein treatment, with regard to the capacity of purging of a number of different cell types.

In accordance with the above results, the use of casein may be anticipated to be beneficial for improving the recovery of stem cells when purging cancer cells from blood and bone marrow, since the purging results in 20–40% loss of progenitor cells.[18] Furthermore, when enriching cancer cells from blood or bone marrow by removing non-tumor cells with M-450 CD45 beads, some loss of cancer cells has been observed[22]. This loss may be reduced by using hydrophilic beads and/or casein.

5.2.5. A New Method for Detaching Beads from Isolated Cells. Recently, a completely new approach to the positive separation of target cells has been developed by establishing a reversible bond between the beads and the antibody system directed against the target cell.[2] After the cells are isolated by use of the attached beads, the bonds between the antibodies and the beads are reversed under mild conditions, thereby liberating the target cells in a nondestructive manner. The principle is illustrated in Figure 8.

Aminophenyl boronic acid is covalently attached to the surface of the beads. The beads are incubated with the monoclonal antibody, which will become attached to the beads through interaction between the boronic acid group on the beads and the carbohydrate units on the Fc part of the monoclonal antibody. By this method a favorable orientation of the antibodies to the bead surface is obtained, leading in turn to a highly efficient and reliable binding to the target cells. After selective isolation of the target cells, sorbitol is added. Sorbitol has a very high affinity for the hydroxyboryl group and will therefore replace the antibody on the beads, which in turn release the cells after less than 2 hours of gentle mechanical treatment at 20°C. This method works well both with IgG and IgM monoclonal antibodies, and have been used directly on peripheral blood and on mononuclear cells PBMC) obtained from buffycoat by gradient centrifugation. The method has been applied for isolation of pure $CD19^+$ (B), $CD4^+$ (T_4), $CD8^+$ (T_8), $CD56^+$ (NK) and $CD14^+$ (monocytes) cells.[83] Recovery of the specific cell type after purging and sub-

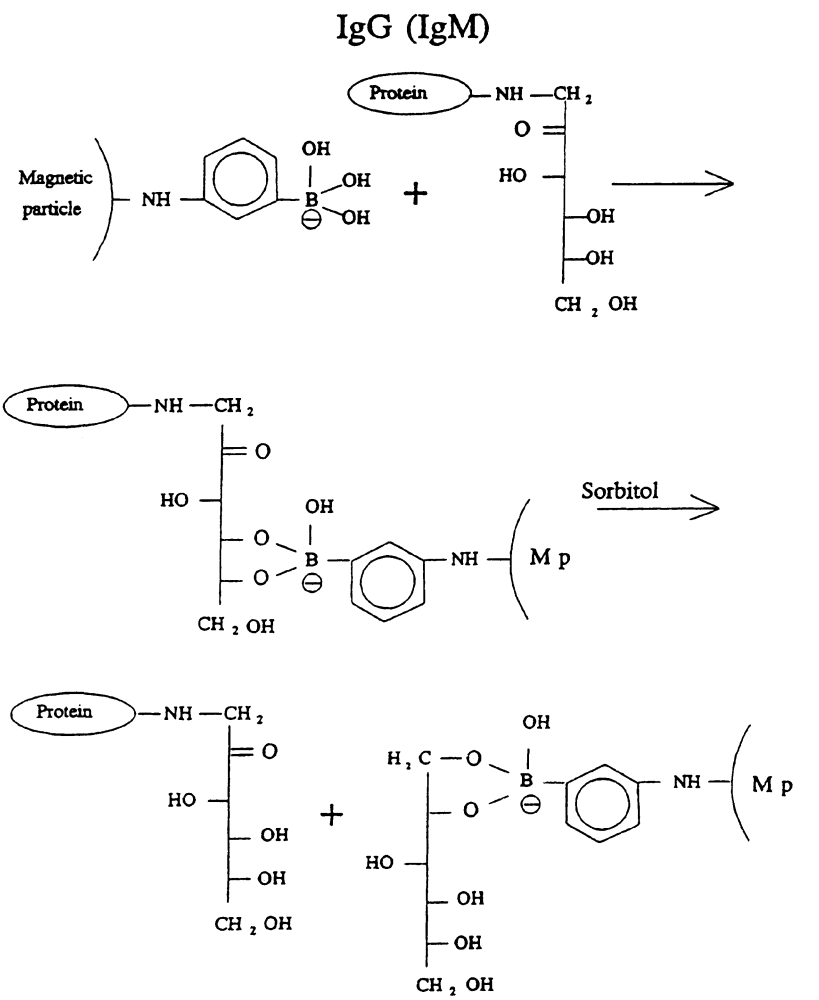

Figure 8. Positive separation by use of magnetic beads with aminophenyl boronic acid groups coupled to the surface.

sequent detachment of beads is with PBMC 70–90% for $CD19^+$, $CD4^+$ and $CD8^+$ cells and 50–60% for $CD56^+$ cells, which is a very good yield compared to previously published results.[32] In all cases, the purity of the isolated cells is greater than 95%. Flow cytometry was used for identification of lymphocyte subsets. The functional properties of the $CD56^+$ cells were investigated after immunomagnetic separation. It was found that the $CD56^+$ cells preserved their functional characteristics, as measured by cytotoxic activity against the NK sensitive K562 cells. Of special interest is the preparation of free monocytes by this method, since it has been difficult to obtain monocytes in pure form due to the phagocytic property of these cells. Preliminary experiments show a recovery of about 50% with regard to $CD14^+$ cells.

A problem with the aminophenyl boronic acid beads is its high non-specific binding of unwanted cells, especially B cells and monocytes. Attempts have been made to reduce the non-specific adhesion of cells by pre-treating the beads with bovine serum albumin BSA) as blocking agent. However, the reduction of non-specific binding which is attainable using BSA, is still too low. It was found that such non-specific binding may be tremendously reduced by using casein as blocking agent. When BSA is used for pretreatment of the beads, monocytes and B cells have to be removed before the isolation of the target cells, otherwise B cells and monocytes will contaminate the isolated cells. After pretreatment with casein, however, there was no need for the removal of B cells and monocytes before their isolation.

6. APPENDIX

6.1. Theory of Gradient Centrifugation

Removal of the excess of magnetic beads by centrifugation is accomplished using two (or three) liquid layers of appropriate density in the centrifuge tube. The beads have a higher density than the target cells with rosetted beads, since the volume of (the lower density) cells constitute most of the volume of the cell/bead complex. The density of the cell/bead complex may be estimated from the following relations between mass (m) volume (V) and density (ρ), using indices "b" for beads, "c" for cells and "cb" for a cell with n beads attached:

$$m_{cb} = m_c + n \cdot m_b = \rho_c V_c + n \cdot \rho_b V_b$$

$$V_{cb} = m_{cb} / \rho_{cb} = V_c + n \cdot V_b$$

The density of the cell/bead complex is then given by:

$$\rho_{cb} = \frac{\rho_c V_c + n \rho_b V_b}{V_c + V_b} \tag{6}$$

To illustrate, the M-280 beads have a radius of 1.4 μm and density ρ_b = 1.5 g/cm^3. The radius of the PM1 cells is 7.4 μm and their density is assumed to be equal to that of the lymphocytes, ρ_c = 1.07 g/cm^3. The corresponding densities of some cell/bead complexes are given in Table 4.

From the table it can be seen that, if a liquid with density larger than about 1.2 g/cm^3 is placed in the bottom of the centrifuge tube, cells with attached beads will not enter this layer. It may also be advantageous to insert an intermediate liquid layer with a density that will promote separation of cells and beads.

If two liquid layers are used, the centrifugation time (Δt) necessary to assure complete separation of beads and cell/bead complexes may be calculated from the expression for sedimentation velocity in a centrifugal field. Assuming that Stokes law is valid, integration gives:

$$\Delta t = \frac{9\eta \ln(x_2 / x_1)}{2 r_{cb}^2 \omega^2 (1 - \rho_{cb} / \rho_b)} \tag{7}$$

Table 4. Densities of PM1 cells and lymphocytes with varying number of rosetted beads

Type of cell	Number of beads per cell	Density of the cell/bead complex (g/cm^3)
PM1	6	1.086
	10	1.098
	20	1.122
Lymphocytes	6	1.174
	10	1.220

where r_{cb} is the radius of the cell/bead complex, η is the liquid layer viscosity, ω is the angular velocity and x_1 and x_2 are the distances from the axis of rotation of the upper liquid surface and the interface between the two liquids, respectively. If three liquid layers are used, two equations with the appropriate values of densities and distances must be applied.

6.2. Isolation of Breast Cancer Cells by IMS and Gradient Centrifugation

Various numbers of PM1 breast cancer cells (generously provided by Dr. G. Kvalheim, The Norwegian Radium Hospital, Oslo, Norway) were added to 10^7 / ml peripheral blood mononuclear cells (PBMC) obtained from buffycoat. Several mAbs that bind specifically to different epithelial surface antigens are available. In these experiments 9189 mAb (IgG, panepithelial 42 kDa glycoprotein, Baxter Munich, Germany) was coupled directly to M-280 beads or to M-280 beads coated with rat anti-mouse. The bead concentration was 3×10^7 / ml. The cell/bead suspension was incubated at 4 °C for 30 minutes. After incubation, the cell/bead-suspension was diluted and placed on a magnet, and the rosetted cells were collected. The tumor cells were visualized either directly or after gradient centrifugation.

So far, Optiprep (Nycomed Pharma, Oslo, Norway) has proven to give the best gradients. Optiprep contains 60% aqueous iodixanol with a density of 1.32 g/cm^3 at 20 °C and an osmolality of 260 mOsm. When Optiprep is mixed with PBS-buffer, isoosmotic solutions could be obtained. The layers used consisted of 40% iodixanol (density 1.213 g /cm^3) at the bottom of the centrifugation tube, 15% iodixanol (density 1.079 g /cm^3) as an intermediate layer and an upper layer consisting of the cell suspension mixed with iodixanol to give a concentration of 10% iodixanol (density 1.052 g/cm^3). With this system, an intermediate layer of target cells with rosetted beads was formed. The excess of free beads was collected in the bottom layer, and, optionally, non-target cells were gathered in the top. Optimum centrifugation speed was 395 g in 5 minutes with a slow retardation (1000 rpm/4 minutes). It has also been observed that non-specific binding of PBMC was reduced with this method.

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