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Novel magnetic microspheres on the basis of poly(vinyl alcohol) as affinity medium for quantitative detection of glycated haemoglobin

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Abstract

A water-in-oil suspension cross-linking technique using poly(vinyl alcohol) as polymer phase to prepare novel magnetic microbeads is described. By dispersing a conventional Fe_3O_4 pigment in the polymer phase and subsequently suspending the mixture in a vegetable oil phase with a defined viscosity, spherical magnetic microspheres are obtained. Bead sizes ranging from 1 to 50 μm and above can be obtained by exploiting well defined preparation parameters such as polymer concentration and oil and polymer viscosity. The performance of the magnetic matrices for the separation and quantification of glycated haemoglobin was tested using a *m*-aminophenylboronic acid matrix. The feasibility of this detection method for blood sugar diagnosis is discussed using a commercial column test kit for comparison.

1. Introduction

The introduction of magnetic microcarriers in the 1970s can certainly be regarded as one of the highlights in the area of biosciences. Since then, they have occupied a broad variety of applications. The initial work featured the incorporation of a ferrofluid into a polymer such as agarose [1], polyacrylates [2], agarose–polyaldehyde [3], polyglutaraldehyde [4] and polyacrolein [5]. These microspheres were mainly designed for cell labelling and cell separation. Further, magnetic microspheres prepared from starch [6] and serum albumin [7] using a "phase-emulsion polymerization" were employed for drug targeting and as drug carriers. An elaborate technology, which has now been commercialized, was introduced by Ugelstad et al. [8] using acrylates and polystyrene microspheres in which iron(III) oxide is incorporated using a swelling–oxidation process. The preparation and application of magnetic poly(vinyl alcohol) (PVA) microspheres in which an Fe_3O_4 colloid is incorporated is the subject of this paper. The microspheres are prepared by suspending a liquid PVA–Fe_3O_4 emulsion in a vegetable oil phase characterized by a defined viscosity. Compared with previously described emulsion polymerization and suspension cross-linking techniques, the preparation technically represents an extremely fast and experimentally simple method which does not require initiators, stabilizers or any other hazardous solvents.

To study the performance of the new magnetic microspheres, glycated haemoglobin (GlyHb), which is related to the blood sugar level, was

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determined by using *m*-aminophenylboronic acid (APBA)–PVA beads which specifically bind GlyHb. In order to evaluate the practicability of the magnetic bead technique with regard to a possible routine diagnostic test, comparative tests were carried out using a commercial APBA agarose column test kit.

2. Experimental

2.1. Materials

PVA with different molar masses (M_n 23 000–224 000) was obtained from Hoechst (Frankfurt, Germany). Prepacked APBA agarose columns were obtained from Merck (Darmstadt, Germany). Bayferrox 318 Fe_3O_4 pigment was supplied by Bayer (Leverkusen, Germany). Dynabeads M-280 were obtained from Dynal (Hamburg, Germany). Azoalbumin was purchased from Sigma Chemie (Deisenhofen, Germany). Standard haemolysed EDTA–blood was supplied as a pooled specimen from the University Hospital, Aachen, Germany. All other chemicals, of analytical-reagent grade, were obtained from Fluka (Neu-Ulm, Germany).

2.2. Apparatus and operating procedures

Viscosimetric measurements were carried out using an RV3 rotating viscometer (Haake, Karlsruhe, Germany). A Beckman DU-7 spectrophotometer (Beckman Instruments, Munich, Germany) was used for the photometric determinations. Bead sizes and bead size fractions were determined with the aid of a laser scattering apparatus (FACstar; Becton-Dickinson, Heidelberg, Germany).

Scanning electron microscopy (SEM) was performed with an SEM 515 instrument (Philips, Hamburg, Germany) with a tilt angle of 45° and electron energies of 10 kV. Sputtering with gold was effected in an SCD 030 sputtering device (Balzer, Lichtenstein), giving coatings of about 30 nm.

2.3. Preparation of magnetic PVA microbeads

Magnetic PVA microspheres were prepared by applying a suspension cross-linking procedure according to the aforementioned method [9]. Briefly, 4.3% (w/w) Bayferrox and 16.6% (v/v) 3 *M* HCl were added to a 5% (w/v) aqueous PVA solution (M_n 224 000). The mixture was sonicated for 1 min in an ultrasonic bath (Branson 2200, 120 W) and then added to a threefold volume (relative to the PVA phase) of a vegetable oil with a viscosity of 130–190 mPa s at 20°C. Suspension cross-linking was carried out at 700 rpm using an IKA RE 166 laboratory stirrer (Janke und Kunkel, Staufen, Germany). Immediately after starting the suspension process, 4.7% (v/v, relative to the PVA phase) of a 25% glutardialdehyde solution was added. Cross-linking proceeded for a further 1 min. The microspheres were isolated from the oil phase by centrifugation for 1 min at 9000 *g*. The beads were then successively washed with *n*-hexane and 2-butanone. Intensive washing with several portions of water then followed for several hours.

2.4. Coupling procedures to PVA microspheres

A 300-mg amount of PVA microspheres was suspended in water for immobilization of azoalbumin to PVA beads. After the suspension had settled for 24 h, 1-ml portions of gel were dehydrated by successively incubating them in 5 ml of acetone–water mixtures (1:3, 1:1 and 3:1, v/v) and finally with anhydrous acetone. Activation with 2-fluoro-1-methylpyridinium toluenesulfonate (FMP) was performed according to the previously described method [10]. Coupling was performed by adding 2 ml of phosphate-buffered saline (PBS) (pH 7.2) containing 15 mg of azoalbumin, with reaction for 20 h at room temperature. The protein content in the supernatant was determined spectrophotometrically at 560 nm.

To couple APBA, 0.5 ml of PVA beads (M_n 224 000; size range 10–25 μm) was first activated with 1,4-butanediol diglycidyl ether (BDE) and FMP according to the previously described meth-

ods. Activation with epichlorohydrin (ECH) and 1,6-hexamethylene diisocyanate (HMDI) was carried out using the method described elsewhere [12]. Before activating the beads with FMP and HMDI, they were dehydrated with acetone–water following the above-described procedure. Coupling of APBA was performed as follows: 1 ml of 0.5 M potassium phosphate buffer (pH 8.0) containing 15 mg of APBA was added to the ECH- and BDE-activated gels and the matrix was shaken for 20 h at 35°C. The FMP-activated gels were incubated for 20 h at ambient temperature with 1 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 15, mg of APBA. Coupling of APBA to HMDI gels was carried out by adding 2 ml of dimethyl sulfoxide (DMSO) in which 0.1% (v/v) tetrabutyltin and 15 mg of APBA were dissolved. The mixture was shaken for 6 h at 30°C.

2.5. GlyHb test procedure

Determination of GlyHb using prepacked 0.5-ml APBA agarose columns (0.6 cm I.D.) was carried out according to the supplier's instruction [13]. A 50-μl volume of pooled EDTA–haemolysate was applied for the determination of the percentage content of GlyHb and 200 μl of a haemolysate to measure the absolute GlyHb binding capacities. Tests were performed at 20–21°C. Tests with the magnetic PVA microspheres were performed in standard glass tubes (5 cm × 0.5 cm I.D.) as follows: after adding 0.5 ml of wash buffer (0.25 *M* ammonium acetate–0.05 *M* $MgCl_2$, pH 8.0) and 50 μl of haemolysate to 0.5 ml of magnetic beads, the suspension was slowly rotated for 2 min. The magnetic fraction was separated by applying a conventional hand magnet for 30 s and the supernatant was aspirated. The beads were washed twice with 2 and 3 ml of wash buffer and the supernatant was aspirated after magnetic separation. The absorbance of the collected aspirated fractions was measured at 414 nm. The bound GlyHb was subsequently eluted from the magnetic beads by adding 3 ml of elution buffer (0.1 *M* Tris–0.2 *M* sorbitol, pH 8.5). After magnetic separation, the supernatant was aspirated and read at 414 nm. The percentage of GlyHb was calculated by using the following equation [13]:

$$\text{GlyHb}\,(\%) = 3.0 \cdot A_2 \cdot 100/5.55 \cdot A_1 + 3.0 \cdot A_2$$

where A_1 represents the absorbance of the unretained non-GlyHb fraction and A_2 that of the GlyHb fraction.

The bead suspension and columns can be regenerated with 0.1 *M* acetic acid.

3. Results and discussion

3.1. Magnetic PVA microspheres

The preparation procedure for the synthesis of magnetic microspheres consists of the following steps:

(1) preparation of a PVA solution with a defined concentration;

(2) addition of a defined amount of HCl and emulsifying a defined amount of Fe_3O_4–colloid in the PVA phase;

(3) suspension of the PVA–Fe_3O_4 phase in a vegetable oil using a set stirring speed;

(4) addition of a defined amount of glutardialdehyde; and

(5) washing with hexane, 2-butanone and water.

The above steps unambiguously demonstrate that the procedure represents a very rapid and simple technical process which, depending on the preparation scale, requires only 5–15 min to prepare the basic magnetic microparticles. Hence, from the experimental point of view, the described PVA method is a much simpler experimental method than previously described techniques [2–4,8,14] which normally require up to 48 h of preparation. In addition, the use of chlorinated and other hazardous aromatic solvents can be avoided.

The morphology, bead size and bead size range of the PVA beads prepared by the suspension cross-linking technique are shown in the scanning electron micrographs in Fig. 1. As can be seen, the beads have a smooth surface morphology. This is a prerequisite for biocom-

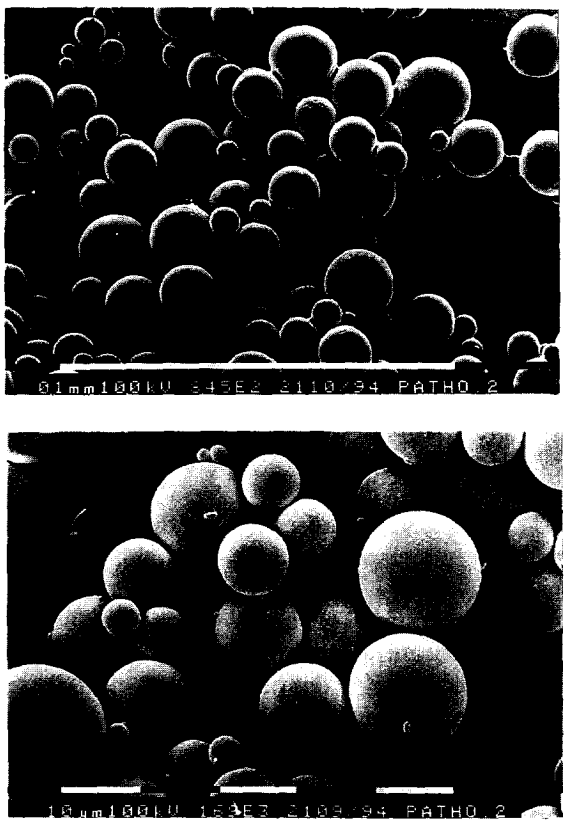

Fig. 1. Scanning electron micrographs of PVA microspheres prepared by the suspension cross-linking technique. (Top) PVA (M_n 224 000) cross-linked with 4% (v/v) glutaraldehyde; (bottom) enlargement of (a). Bars indicate (a) 100 μm and (b) 10 μm.

patibility and blood compatibility in particular [15]. The physical structural parameters are one of the major features of magnetic microspheres; these include porosity, mechanical stability, size and specific density. The PVA technique allows optimum adaptation to diverse requirements by varying the following parameters: molar mass of PVA, concentration of PVA solution, cross-linking concentration, viscosity of PVA phase and viscosity of the oil phase [9].

In general, the bead porosity increases with increasing molar mass of the polymer, which is accompanied by a parallel decrease in the specific density [16]. The validity of this relationship can be demonstrated by comparing the amount of azoalbumin bound to FMP-activated

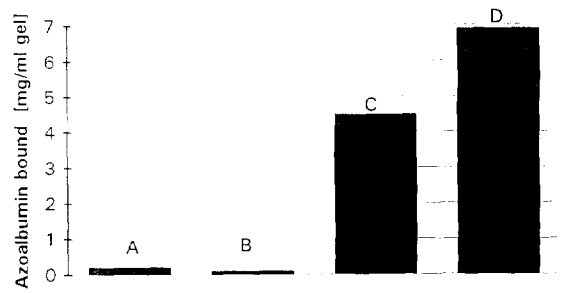

Fig. 2. Amount of azoalbumin bound to FMP-activated PVA carriers. Columns A–D represent the PVA samples with the following molar masses, polymer concentration and most frequent size fraction: (A) M_n 23 000, 10% (w/v), 15 μm; (B) M_n 84 000, 10%, 38 μm; (C) M_n 84 000, 5%, 22 μm; (D) M_n 224 000, 5%, 46 μm. Cross-linker concentration in all cases: 2.5% (v/v).

PVA beads with different molar masses (Fig. 2). The test shows that there is a distinct increase in protein binding with increasing molar mass and decreasing polymer concentration. Beads prepared from a 10% PVA solution with a molar mass of 23 000 (Fig. 2, column A) and a 10% PVA solution having a molar mass of 84 000 (column B) reveal extremely low protein uptakes. In contrast, PVA with a molar mass of 224 000 (column D) and 84 000 (column C), the latter having a polymer concentration of 5% (w/v), show a substantial concentration of bound azoalbumin amounting to 6.9 and 4.5 mg/ml gel, respectively. Owing to the different beads sizes which result from the diverse viscosities of the polymer phases (see Fig. 3), the established relationship between molar mass and protein binding capacity can only be deduced qualitatively in the present test. The basic validity of the above finding is confirmed if one compares the visual staining depth of the beads: PVA samples with M_n 224 000 and 84 000 (5% concentration) show an intense yellow colour, whereas the other two specimens show only a very slight yellowish staining.

As all other test parameters and the cross-linking concentration in particular were kept constant, the molar mass and polymer concentration as determining parameters for the porosity of the beads become obvious. The reason for this can mainly be ascribed to the extended

molecular coil of the polymer, which results from an increasing molar mass and decreasing polymer concentration in solution. In addition to the porosity and its concomitant ligand binding properties, the application of magnetic microspheres is predominantly governed by the bead size. Small bead sizes with a range of 1–10 μm are preferred for the application of magnetic microspheres in protein separation and bioassays and cell separation and labelling. The reason for this is that they offer an appropriate surface area to bind sufficient amounts of bioligands. Another advantage of small beads sizes is their ability to sustain in aqueous suspension, thereby permitting reaction kinetics of a "quasi-homogeneous" solution. With regards to this property, the diverse preparation parameters allow the procurement of a favourable adjustment of the PVA density.

Depending on the preparation conditions, the specific gravity ranges from 1.05 to 1.2 g/cm^3. This makes possible a very long suspension time in aqueous media. Comparative tests with 1 ml of standard Dynabeads M-280 and 1 ml of magnetic PVA suspension (bead size 2–4 μm, 30 mg solid PVA/ml) in PBS (pH 7.2) revealed a sedimentation time of about 3 h for the Dynabeads and about 12 h for PVA beads.

Using the diverse modifiable experimental parameters, the bead sizes of PVA can be adjusted within a broad range from 1 to 1000 μm. Small sizes, particularly those between 1 and 10 μm, are preferentially used in bioassays [17]. To obtain the different size ranges, one can exploit the viscosities of both the oil and the PVA phases [9]. Fig. 3 shows the relationship between the bead size and the viscosity of the PVA solution at a pre-set viscosity of the oil phase and stirring speed. The tests reveal that on increasing the viscosity of the aqueous polymer phase, the PVA beads become larger. In the present test, the different viscosities were obtained by selecting molar masses of 23 000, 48 000, 84 000 and 224 000. The relevant polymer concentration was 10% (w/v), except for PVA of M_n 224 000, where concentrations of 5% and 2.5% were used.

In addition to these parameters, defined viscosities of the polymer phase can also be adjusted by varying the temperature. The relationship between the temperature of the oil phase and the bead size is depicted in Fig. 4. The results indicate that by increasing the temperature from 18 to 75°C, which corresponds to viscosities from 20 to 190 mPa s, the beads sizes increase from 12 to 37 μm (most frequent diameter fraction). The main feature of PVA when compared with previously described polyacrylates [8] is that PVA is basically very hydro-

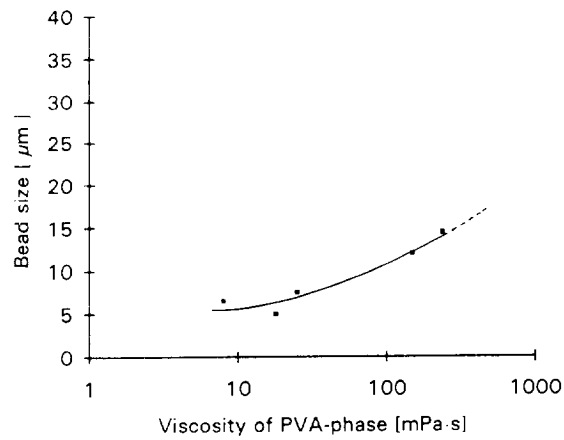

Fig. 3. PVA microsphere bead size as function of the viscosity of PVA solution. Symbols represent the most frequent bead size fraction within the bead size distribution measured by laser scattering experiments (see Experimental).

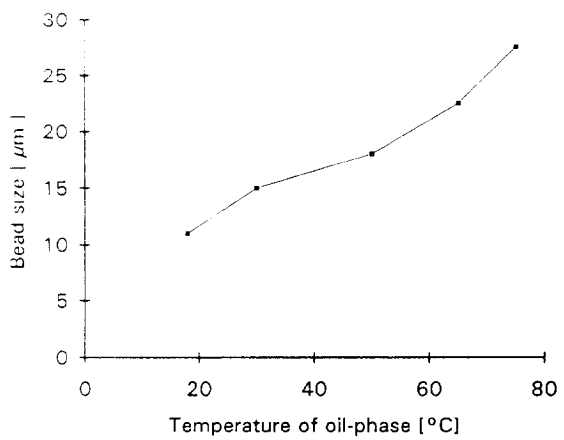

Fig. 4. Bead size of PVA microspheres as function of the oil-phase temperature. PVA M_n, 224 000; polymer concentration, 5% (w/v); cross-linker concentration, 4% (v/v). Symbols: most frequent bead size fraction (see Fig. 3).

philic and consequently does not tend to unspecific protein adsorption. This has been confirmed by tests with Sepharose CL-6B, Sephacryl S-200 (both from Pharmacia–LKB, Uppsala, Sweden), silica gel type GF (Sigma, Chemie), Synsorb (Chembiomed, Edmonton, Canada) and Fractogel TSK 75 (Merck) using fibrinogen as test protein. Under identical test conditions, these matrices adsorb 4–15 times more fibrinogen when compared with PVA beads [18].

The influence of the stirring speed on the bead size is shown in Fig. 5. From the results one can conclude that under constant experimental conditions (e.g., viscosity of oil phase, polymer concentration, stirrer geometry, design of reaction vessel, cross-linker concentration), the bead size decreases with increasing stirring speed. This is in agreement with tests described for the preparation of albumin microspheres [19].

3.2. GlyHb determination using APBA carrier

APBA has been used for the specific adsorption of glycated proteins and nucleic acids [20–23]. The separation principle is based on the specific interaction of a *cis*-diol-containing compound with phenylboronic acid to form a five-ring complex which can be specifically dissociated with sorbitol. The same separation principle can also be used specifically to separate and determine GlyHb levels in blood. GlyHb is formed by a non-enzymatic glycation of Hb by glucose, which forms a Schiff base adduct with diverse amino groups of the Hb tetramer. This linkage subsequently undergoes an Amadori rearrangement to form a stable GlyHb [24]. The GlyHb formation is directly correlated with the long-lasting chronic diabetes status.

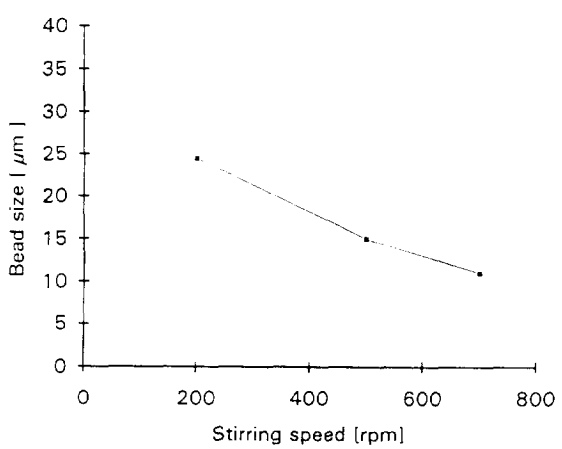

Fig. 5. Effect of stirring speed on the particle size of PVA microspheres. PVA M_n, 224 000; polymer concentration, 5% (w/v); cross-linker concentration, 4% (v/v). Symbols represent the most frequent bead size fraction (see Fig. 3).

Differently activated magnetic microspheres to which APBA was attached were applied to evaluate the performance of the magnetic beads in comparison with a commercial test kit using a prepacked APBA–agarose column. In Table 1, the GlyHb test results using PVA and the commercial APBA agarose column are summarized. Although the magnetic bead determination method is not yet fully standardized, the percentage measurements obtained with the two methods correspond relatively well and reveal GlyHb concentrations of 5.7% and 6.4%. The relative standard deviations are 9.1% for the column method and 11.4% for the magnetic bead procedure.

To evaluate the detection range of the PVA carriers and the agarose column, 200 μl of haemolysate were applied to the matrices. The amounts of GlyHb recovered from the 200 μl of a haemolysate were 0.4 mg for PVA and 0.28 mg for the agarose matrix (Table 1), indicating that PVA beads offer a broader detection range than the APBA–agarose.

The influence of the buffer pH and temperature on the performance of analogous APBA agarose columns have been described by Klenk et al. [25]. These tests showed that the buffers and buffer pH (wash buffer, pH 8.0; elution buffer, pH 8.5) used for the present investigations provide optimum conditions for the test procedure. With regard to the influence of temperature, Schmid and Vormbrock [26] demonstrated that within the range 20–25°C, the GlyHb content increases only by 0.1% per 1°C temperature increase. Hence the present test procedures conducted at 20–21°C do not require any temperature correction.

From the experimental point of view, the magnetic separation technique reveals distinct

Table 1
Comparison of the procedures for the determination of the percentage of GlyHb and the absolute amount of GlyHb in pooled haemolysates using APBA magnetic PVA and a prepacked APBA–agarose column

Sample	GlyHb (%)[a]	S.D.	R.S.D. (%)	GlyHb bound (mg/ml gel)[b]	Test time (min)
Isocyanate-activated magnetic PVA	5.7	0.65	11.4	0.40	8
FMP-activated magnetic PVA	–[c]	–	–	–[c]	8
BDE-activated magnetic PVA	–[c]	–	–	–[c]	8
Prepacked agarose column	6.4	0.58	9.1	0.28	20

[a] Determined using 50 μl of a haemolysate; mean of five determinations.
[b] Determined using 200 μl of a haemolysate; mean of five determinations.
[c] Not detectable.

advantages over the column chromatographic method. The separation of a 50-μl haemolysate specimen can be carried out within 8 min using the magnetic method, whereas the column technique requires ca. 20 min. Apart from the aspect of time saving, conventional glass test-tubes can be used for the magnetic bead technique, whereas the column technique requires specially manufactured plastic columns fitted with top and bottom frits.

When comparing the different activation/coupling modes (Table 1) which were employed to attach the APBA to the PVA matrix, the test results reveal that the isocyanate coupling method is the only method giving satisfactory results. In contrast, neither coupling via FMP, generally an effective method for protein coupling [27], nor the matrix coupled via BDE showed any substantial GlyHb binding. The latter finding also applies to the activation/coupling using ECH (results not shown), which also does not bind any detectable protein. The results corroborate recent findings, namely that ligands (e.g., oligosaccharides) which are soluble in aprotic solvents such as dimethylformamide or DMSO can be favourably linked to these types of activated matrices [12]. The poor binding results obtained with FMP- and BDE-activated matrices are obviously caused by the insufficient nucleophilicity of the aromatic amino group of the APBA, which prevents binding to the support.

Apart from the described column separation method applied here for comparison purposes, a number of other methods for the determination of GlyHb have been described. The most important include ion-exchange chromatography, HPLC, fructosamine assay, electrophoresis and immunoassay. The advantages and disadvantages have been discussed in detail elsewhere [23,25]. With regard to the magnetic method, one can conclude that all methods, except the fructosamine assay, are time consuming and require either costly reagents (immunoassay), cumbersome and standardized analytical measures (electrophoresis) or expensive apparatus (HPLC). Further, the affinity methods applied here encompass the whole amount of the glycated Hb, in contrast to the ion-exchange method which only allows the detection of β-terminal glycated specimens. Hence the affinity method may provide more relevant data with regard to precise diagnostics [26].

In view of these findings and considering the high clinical relevance of long-lasting increased

blood glucose levels in diabetic patients, methodologically improved quantification systems are desired. The determination of GlyHb using APBA magnetic microspheres can certainly be regarded as an interesting alternative to currently used methods and could thus replace established methods in blood sugar diagnosis after appropriate standardization.

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