Preparation Characterization and Blood Compatibility Studies of Silk Fibroin / Gelatin / Curcumin based Injectable Hydrogels

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Abstract

Background

Hydrogel is a structure with three-dimension that have the potential to absorb and retain water inside of it. Currently hydrogels made from natural biopolymers are preferred in the discipline of biomedicine due to their nature of blood compatibility, adhesion of platelets and protein binding, ease of administration and delivery of ingredients to the place of action. The purpose of this study is preparing blood compatible injectable hydrogels from naturally obtained polymers of biomaterials in a combination of different proportion, characterizing and analyzing its physiochemical blood compatibility and morphological structures.

Methods

Three different compositions of gelatin, dialyzed SF, curcumin and N, N methylene bisacrylamide (MBA) in different groups coded by IVA, IVB and IVC were used. The combinations were evenly mixed on a magnetic stirrer. After an hour of the gelation process it has been kept under refrigerator at 4°C. For characterization and biocompatibility studies of hydrogel swelling test and biodegradation analysis, SEM, FTIR, *In-vitro* coagulation tests, total serum albumin and cholesterol level analysis were applied,

Results

Injectable hydrogel successfully made from the combinations of SF/GE/CU/ in the presence of a cross linker MBA and the result from physiochemical biocompatibility and morphological characteristics study were confirmed that natural biopolymers used in this study are a candidate for biomedical applications.

Conclusion

The result confirmed that the composition coded by IVC was identified the most stable composition and suitable in its morphological structure with excellent blood compatible nature recommended for further biomedical applications.

Introduction

Hydrogel is a biological fluid or a structure with three-dimension that have the potential to absorb and retain water inside of it [1]. Hydrogels are suitable for the formation of polymer networks via crosslinking chemicals or by the influence of physical impact or enzymatic actions which grants for the hydrogel to allow the movement of particles or materials loaded on it freely [2]. Nowadays hydrogels are believed to be very attractive biomaterials in the area of biomedical applications starting from tissue engineering to drug delivery with their high quality of properties from its nature [3].

Equilibrium swollen state, morphological structure of networks and defined chemical structure of the hydrogel determines the loading and transport of materials, mechanical strength and adaptation of the new environment [4], besides, the huge volume of water that they can hold on and their delicate consistency hydrogels made from naturally obtained polymers have favorable properties, such as biocompatibility, biodegradability and imitating extracellular matrix of the host living cells [5].

Hydrogels as a biopolymer form a network where the crosslinking chains are bonded either covalently or make a non-covalent interaction, the formed cross-link of the hydrogels grants to be a permeable network that allows the movement of molecules like loaded drugs, important nutrients and oxygen through the intertwined structure. [6, 7].

Hydrogels made from natural biopolymers nowadays preferred in the discipline of biomedicine due to their nature of blood compatibility, adhesion of platelets and protein binding [8], ease of administration and delivery of ingredients to the place of action [9]. Naturally obtained biomaterials like silk fibroin (SF), gelatin (GE) and curcumin (CU) have found a countless application in the area of regenerative medicine including tissue engineering due to their biodegradability and blood compatibility [10], more remarkably naturally made polymers of hydrogels exhibit high potential as drug delivery units due to their greater biological values [11].

Silk fibroin (SF) adequately utilized for the production of biopolymers which widely used for biomedical applications in different forms such as hydrogels [12], scaffolds, films, particles and sponges [13], all this forms experimentally confirmed to be used for the purpose of better biomedical units due to its remarkable properties of hemocompatibility, interaction with cells, biodegradability, biocompatibility and low toxicity [14], water absorption capacity and low immunogenicity [15]. It is confirmed that silk fibroin as a natural biomaterial can be a potential raw material due to its adaptable properties with natural physiology and capability to promote cell formation that makes it to be preferable in the study of tissue reconstruction in age related ocular disease particularly glaucoma [12].

Curcumin (1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione) a turmeric root a bright yellow chemical produced naturally as curcuma longa composed of different chemicals playing a pivotal role acting as anti-microbial activities, antioxidant anti-inflammatory, antineoplastic, wound-healing, and additionally the potential chemotherapeutic properties best suits for biomedical applications which improves drug delivery system[16].

Gelatin ($C_{102}H_{151}N_{31}O_{39}$) is obtained and prepared by passing in the process of partial hydrolysis on the natural collagen [17]. It has an exceptional gelling behavior in addition common functional characteristics with proteins, thus it has been broadly applicable in many manufacturing industries for instance in the food processing, cosmetics and medicine fields [18]. Gelatin have a wide spread application because of its toxin free nature some of the area are acting as major food items, applicable in pharmaceutical and for making biomedical devices as well. Transparency is the major properties of gelatin which contrasts with most foods more or less turbid [19].

According to the survey done by researchers, the characteristic features of hydrogels including biodegradability, swelling property and biocompatibility can be controlled and modified by the type of polymers and preparation procedures[20], rapid hydrogel formation as well as gelation capability were regulated by the association of polymeric network and functional groups of the polymers used [21]. It is also stated by J. Leijten et al. [6], hydrogels as a biopolymer exceptionally forms a network where the crosslinking of chains are bonded either covalently or make a non-covalent interaction [22], the formed cross link of the hydrogels grants to be a permeable network that allows the movement of molecules like loaded drugs, important nutrients and oxygen through the intertwined structure.

This study was designed to prepare injectable hydrogel by a combination of natural biopolymers (SF/CU/GE with N, N' Methylene Bisacrylamide (MBA) as a cross linker) and evaluate its blood compatibility, swelling nature, prolonged degradation and morphological features under *in vitro* conditions in order to recommend for further clinical use.

Materials And Methods

Materials

Bombxy mori cocoons and Curcuma longa (Turmeric) rhizome were obtained from a local market of North Cyprus. Gelatin, Tween 80, Calcium chloride (CaCl2), Sodium triphosphate Pentabasic (Na5O10P3), Ethanol (C2H5OH, 98%) were all purchased from Sigma Aldrich. SnakeSkin® Dialysis Tubing of 3,500 molecular weight cut out membranes was purchased from Thermo Scientific USA. anhydrous sodium carbonate (Na2CO3) and anhydrous calcium chloride (CaCl2) obtained from EMSURE® Merck chemicals in Darmstadt, Germany. All experiments involved the use of deionized water and ultrapure water. Naturally obtained polymers purchased from the local markets are purified according to the standard protocols.

Fabrication Procedures

Silk fibroin Purification

The process of purification was carried out by cutting SF cocoons into smaller pieces and mixing with 0.1M Na_2CO_3 solution into the flask by using electro-spurned magnetic stirrer at the speed of 1.5rpm for three sessions at 70°C. After each session of degumming SF washed by using ultra- pure water and dried for overnight at room temperature, then dissolution carried out by strong electrolyte solution (molar ratio of nH_2O:$nCaC_{l2}$:$nCH_3CH_{20}H$; 8:2:1) with continuous stirring at 70°C. Finally, dialysis of the solution was done by using a snake skin dialysis membrane against deionized water to remove all the strong electrolyte in the solution of silk fibroin finally the already purred solution of silk fibroin was extracted from the dialysate by using syringe and then poured into the bottle [14, 6].

Curcuminoid isolation and extraction

Percolation extraction method was applied by adding acetone to 144 g of curcuma longa powder the process was repeated three times after completing the extraction of curcuminoid the rotary evaporator

was used for concentrating the extract, column chromatography was used for purifying the concentrated extract of curcumin, silica gel (SiO_2) and dichloromethane used as adsorbent. After purification by heating dichloromethane was removed finally the byproduct collected in the form of powder and subjected to thin layer chromatography analysis the setup for column chromatography is done and carried out to isolate the pure curcumin sample finally, pure curcumin samples were collected in test-tubes and stored.

Preparation Of Hydrogels

For preparing hydrogels as indicated in Table 1, gelatin (GE), dialyzed silk fibroin (SF), curcumin (CU), and N, N methylene bisacrylamide (MBA) (as a cross linker) were used in the following protocols.

1. SF/GEL/MBA (IVA): 1.5ml of 3% SF, 5ml of 10% GE and 5µl MBA were mixed evenly on a magnetic stirrer and left to gelate then the hydrogel stored under refrigerator at 4°C.

2. SF/GEL/CU/MBA (IVB): 2ml of 3% silk fibroin, 0.25ml of CU and 5µl MBA were added to 5ml of 10% GE and mixed properly on a magnetic stirrer later left to gelate and kept overnight at 4°C.
3. SF/GEL/CU/MBA (IVC): 2ml of 3% silk fibroin, 0.25ml of CU and 5µl MBA were added to 3ml of 10% GE which were all mixed on a magnetic stirrer. After an hour of the gelation process it has been kept under refrigerator at 4°C and all the samples were freeze dried into a balloon at -20°C.

Table 1
Composition used for hydrogel formation

Code	Gelatin (ml)	Silk fibroin (ml)	Curcumin	MBA
IVA	5ml	1.5ml	-	5µl
IVB	5ml	2.0ml	0.25ml	5µl
IVC	3ml	2.0ml	0.5ml	5µl

Characterization And Morphological Studies Of Hydrogel

Swelling/degradation test

Swelling tests were carried out by immersing the samples in 20ml of PBS (pH 7.4) solution and ABS solution at 37℃ at predetermined time intervals the hydrogel was carefully taken and blotted with filter paper and weighed and returned back to the same medium so that measurement was made to the maximum swelling point. The range of pH was chosen to mimic the human physiological conditions and swelling ratio was calculated by the formula:

Swelling ratio% = $\frac{\mathrm{Ws}-\mathrm{Wd}}{Wd} \times 100\%$

Where Ws = weight of the swollen sample and Wd = initial weight of the sample

Scanning electron microscope (SEM) analysis

Morphological and structural features of injectable hydrogels prepared at static condition were carried out by using scanning electron microscope (SEM, at TUBITAK-MAN using a JEOL/JSM-6510LVF). After the samples were lyophilized the micro-structure were imaged at x100 and x200 magnification at 20kv of acceleration voltage.

Fourier transform infrared (FTIR) analysis

The Fourier transform infrared (FTIR) spectra of tested samples were carried out in the laboratory of Eastern Mediterranean University Northern Cyprus Famagusta using a Perkin Elmer Spectrum 65 FTIR

Blood Compatibility Studies of the Hydrogel

In-vitro **coagulation and fibrinogen activity test analysis**

For *in-vitro* anti-coagulation test analysis STA compact was used. Fresh blood sample collected from the healthy donor in sample collection tubes containing anticoagulant trisodium citrate 0.109M (3.2%) according to the CLSI guidelines and centrifuged at 850 RCF for 10 minutes' blood plasma were taken and sample of hydrogel dropped inside and incubated under static condition of 37°C for about 15 minutes. Prothrombin time, Activated Partial thromboplastin time and fibrinogen analyses were performed [23]. APTT results and PT analysis results were reported in seconds and International Normalized Ratio INR and fibrinogen results were reported as mg/dL.

Total serum albumin and cholesterol level analysis

Fresh blood collected from the healthy donor and sample hydrogel were mixed and incubated for 30 minutes under 37°C then centrifuged by the speed 1500RCF for 10minutes, serum albumin and cholesterol level were measured. serum albumin is a protein in the blood plasma represents almost half of the total proteins ranging in between 3.5g/dL to 5g/dL in healthy persons protein [11]. Cholesterol is a natural substance made by the body around 75% of it is made by liver and is important for normal physiological activities in optimal conditions. A total cholesterol score of 200mg/dl or lower is considered to be optimal [24, 25].

Complete blood count analysis and erythrocyte morphology studies

Automated hematology analyzer was used for complete blood count analysis illustrated in Table 2 (Cell Dyn Ruby, Abbott Laboratories, Diagnostic Division, Abbott Park, IL). Fresh blood collected from the donor and mixed with the sample hydrogel, K_2 EDTA was used as anticoagulant. The samples were mixed by

shaker (300 revolution/minutes) which facilitates the interaction of sample surface and blood cell, for 20 minutes and then the following parameters were analyzed

Table 2
parameters used to examine complete blood count analysis

WBC (White blood cells)	RBC (Red blood cells)
Neutrophil	Hemoglobin concentration
Lymphocyte	Hematocrit (relative volume of erythrocytes)
Mononuclear	Mean Corpuscular (erythrocyte) Volume
Eosinophil	Mean Corpuscular (erythrocyte) Hemoglobin
Basophil	Mean Corpuscular (erythrocyte) Hemoglobin Concentration
	Red Cell (erythrocyte volume) Distribution Width
	Platelet or thrombocyte count
	Mean Platelet (thrombocyte) Volume

Platelet Adhesion Analysis In-vitro

Fresh human blood from the healthy donor was collected and then centrifuged using 100 RCF for 15minutes, platelet rich plasma prepared and sample of hydrogel were immersed into it for about 15minutes under human physiological condition 37°C. Peripheral smear test was used to determine the adhesion morphology, micro-particle platelet formation on the surface of hydrogel by using light microscope with low (100×) and high (400×) magnification power

Statistical Analysis

All data were presented as mean ± standard deviation. Significance difference were performed by a student's t test at a probability level of 0.05 and one-way (ANOVA) used for determining the difference among the groups using Graphpad prism version 8.0.2 software.

Result And Discussion

Swelling kinetics

Swelling nature of prepared injectable hydrogel were tested by using acidic phosphate saline (ABS, pH 1.2 and pH 4.7) seen in Fig. 1b and 1c and basic medium phosphate buffer saline (PBS, pH 7.4) Fig. 1a. The swelling behaviors of samples coded by IVB and IVC swells consistently and have been reached their maximum equilibrium ratio accordingly, sample coded by IVB within 180 minutes and IVC 45minutes in

pH 7.4 PBS solution. Whereas the same sample in ABS of pH 4.7 reaches its maximum equilibrium swelling within 90 minutes for IVB and 45minutes for IVC as well as in ABS solution pH 1.2 for sample IVB within 35 minutes and IVC within 20 minutes, however one of the samples coded by IVA quickly swells up in both ABS and PBS within 10minutes then degrade totally.

The % swelling result in PBS pH 7.4 for IVA, IVB and IVC shown in Fig. 1 are 98%, 1469.2% and 1393% respectively. While in Figs. 2 and 3 gives the % swelling in acid buffer of pH 4.7 for the same sample 145%, 700.4% and 1242.6% respectively and in ABS of pH 1.2 for the same samples were 125%, 1440.9% and 1092.1% respectively.

The result confirmed that the swelling of hydrogels by absorbing massive amount of liquids takes place at the beginning of the experiment and gradually become saturated by expanding the network of hydrogel as more fluid is absorbed which confirm a hydrophilic nature of hydrogel due to the free hydroxyl and amino groups within the polymeric matrix [25]. however, sample IVA swells immediately both in acidic and basic buffer solutions within 10 minutes due to its more amorphous nature and having small amount of silk fibroin composition compared to the other samples in agreement with the study conducted by Terada and his colleagues [26].

In-vitro **coagulation, fibrinogen, total cholesterol and serum albumin analysis of hydrogels**

The anti-coagulant activities of the hydrogel were evaluated by APTT, and PT measurements which were handled for evaluating secondary hemostasis. Values of PT and APTT activation for control citrated plasma and after contact with samples (IVA, IVB & IVC) at a temperature of $37 \pm 1^{\circ}C$ were measured. The result obtained from the test for APTT, PT and INR are all in the standard boundary which is a confirmation of blood biocompatibility of the samples.

Total level of cholesterol and serum albumin of samples coded by IVA, IVB and IVC in comparison with the control blood serum coded by HK found to be in the range of desirable boundaries according to the standard by the National Cholesterol Education Program (NCEP).

A fibrinogen activity test result again confirms the blood compatibility of the samples according to the standard value here indicated in the Table 3.

Table 3
Coagulation, Fibrinogen, total cholesterol and Serum Albumin analysis of Hydrogels (IVA, IVB IVC).

Code	PT (%)	INR	PT (time)	APTT (time)	Total level of Cholesterol (mg/dL)	Albumin (g/dL)	Fibrinogen
Standard	70 to 120%	0.8 to 1.2	11.5s to 15s	23.6s to 35.2s	200	5.18	200 mg/dl to 400 mg/dl
IVA	92%	1.06	13.5sec	35.4s	160mg/dl	4.6g/dl	237 mg/dl
IVB	86%	1.11	14.1sec	37.9s	160mg/dl	4.6g/dl	235 mg/dl
IVC	94%	1.04	13.3sec	34.6s	160mg/dl	4.6g/dl	253mg/dl
HK	101%	0.99	12.7sec	31.7s	160mg/dl	4.6g/dl	253mg/dl

PT = prothrombin, INR = International Normalized Ratio, APTT = Activated partial thromboplastin time IVA, IVB and IVC = are coded samples of hydrogels HK = control blood plasma

Complete blood count analysis

The hydrogels have been immersed into fresh blood with K_2 EDTA as anticoagulant. In all samples (IVA, IVB, IVC) no significance difference was recorded from control (HK) as shown in the Table 4 all the results were under the limit zone. This experiment confirms to the fact that the polymers used for preparing hydrogels were blood biocompatible and don't bring adverse influence on the total nature of erythrocyte, leukocyte and platelets in the normal blood.

Table 4
Total blood count analysis of hydrogel samples coded by IVA, IVB and IVC

Samples Analyzed				
Cells	Control	IVA	IVB	IVC
WBC	5.29 10e3/uL	5.12 10e3/uL	5.10 10e3/uL	5.03 10e3/uL
NEU	1.35	1.78	1.30	1.37
LYM	3.18	2.13	2.99	2.51
MONO	.473	.422	.529	.445
EUS	.228	.265	.219	.637
BASO	.060	.012	.068	.077
RBC	5.37 10e6/uL	5.11 10e6/uL	5.43 10e6/uL	5.36 10e6/uL
HGB	16.0 g/dL	15.4 g/dL	16.2 g/dL	16.0 g/dL
HCT	48.2%	46.0%	49.1%	52.5%
MCV	89.8fL	90.0 fL	90.3 fL	97.8 fL
MCH	29.7 pg	30.2 pg	29.8 pg	29.8 pg
MCHC	33.1g/dL	33.6 g/dL	33.0 g/dL	30.5 g/dL
RDW	11.5%	11.5%	11.7%	13.2%
PLT	248 10e3/uL	194 10e3/uL	252 10e3/uL	296 10e3/uL

Peripheric smear test for in-vitro **platelet adhesion analyses**

For characterizing the hemostatic condition of the hydrogel, *in vitro* peripheric smear test (400× magnification) and Wright-Giemsa stains (400× magnification) erythrocyte morphology analysis was applied as it is shown in the Figs. 2 and 3 the result of the samples from microscope micrograph briefly shows that there is no platelet adhere formation on the surface of hydrogel which makes the hydrogel to be blood compatible and can be used for biomedical applications.

Scanning Electron Microscopy Analysis

The SEM micrograph (100μm) as seen in the Fig. 4 below are the morphology of IVA, IVB and IVC coded hydrogels made from different combinations of silk fibroin, gelatin, MBA, and curcumin. In samples of IVA the number of pores was low and their morphology was rough therefore it could be vital for loading of drugs, IVB and IVC contains visible microspheres with open pores which facilitate transport of drugs and

other important nutrients including oxygen which is in agreement of the study conducted by the researchers [26]. The increased amount of SF with decreasing gelatin and the presence of curcumin as well might have been responsible for the morphological features of hydrogels having better pore structure and well interconnectivity that favors for the drug load.

Fourier Transform Infrared Spectroscopy Analysis

In the hydrogel, the specific groups of SF/GE/CU/MBA are characterized by the absorption band in Fourier transform infrared spectroscopy **(**FTIR) spectroscopy.

In Fig. 5a the absorption band region within 1630.48 cm^{-1} – 1382.6 cm^{-1} is assigned to the amide I, as well as in the Fig. 5b the band region indicated within 1537.41cm^{-1} and 1451.4 cm^{-1} to the amide II absorption mainly from the NH bend in-plane bending vibrations and CN stretching in protein backbone of silk fibroin which is in agreement with the work of Adali and her collogues [27]. The bands assure in the β-sheet confirmation of the samples [27].

Figure 5c confirmed that the peaks 2923.10 cm^{-1} and 2853.42 cm^{-1} were due to CH_3 stretch. Since curcumin was completely mixed into the mixture the stretching vibration could be very limited and the bands disappeared in the complexes of SF [28]. Due to the presence of CO and CN vibration stretching of amide I and amide II spectrum exhibited its characteristics bands at 1636 cm^{-1} and 1558 cm^{-1} which is in agreement with the study of Rafael F.N., and his collogues [29]. The hydrogel showed an absorption band 3300.41cm^{-1}, 3283.94 cm^{-1} and 3284.10 cm^{-1} which were due to OH and NH stretching vibration in all hydrogels.

Conclusion

In the present study, injectable hydrogel successfully made from naturally obtained biopolymers and physiochemical biocompatibility and morphological characteristics were determined. The overall result on the analysis were confirmed that hydrogels made by the combination of silk fibroin gelatin curcumin and MBA as a chemical cross linker were to be candidates for the biomedical applications *in vitro* with excellent blood compatibility and desired morphological characteristics. The morphological structure of samples from scanning electron microscopy analysis also showed that sample coded by IVA have low and rough pores and samples of IVB and IVC contains miscible microspheres with open pores that is vital for biomedical applications of loading and transporting different ingredients including drugs. Therefore, the result of the study indicates that hydrogels made from natural biopolymers become a potential source and suitable matrices with excellent blood compatible nature acting as a useful device in the biomedical applications. Particularly the result confirmed that the composition coded by IVC was identified the most stable composition and suitable in its morphological structure Further studies will be carried out in order to identify the mechanisms of drug delivery systems for different therapeutic activities.

Declarations

Author contributions

Kassahun Akulo: Design, analysis and writing. Terin Adeli: Material preparation, design Conceptualization. Oğuz Han Ebedal: Data collection

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Declarations

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

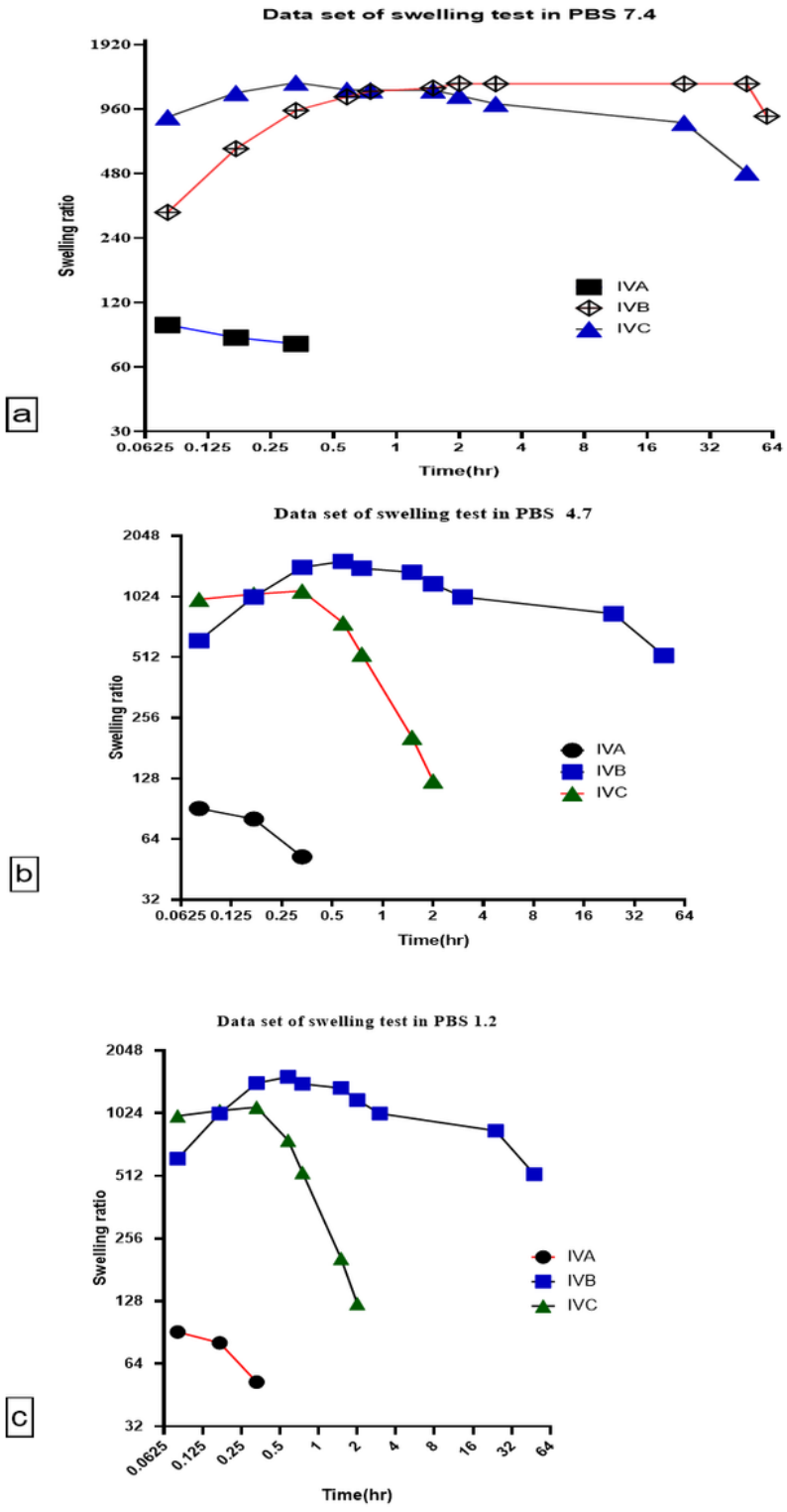

Figure 1

Swelling % hydrogels of sample IVA, IVB, IVC in PBS pH 7.4, 4.7 and 1.2

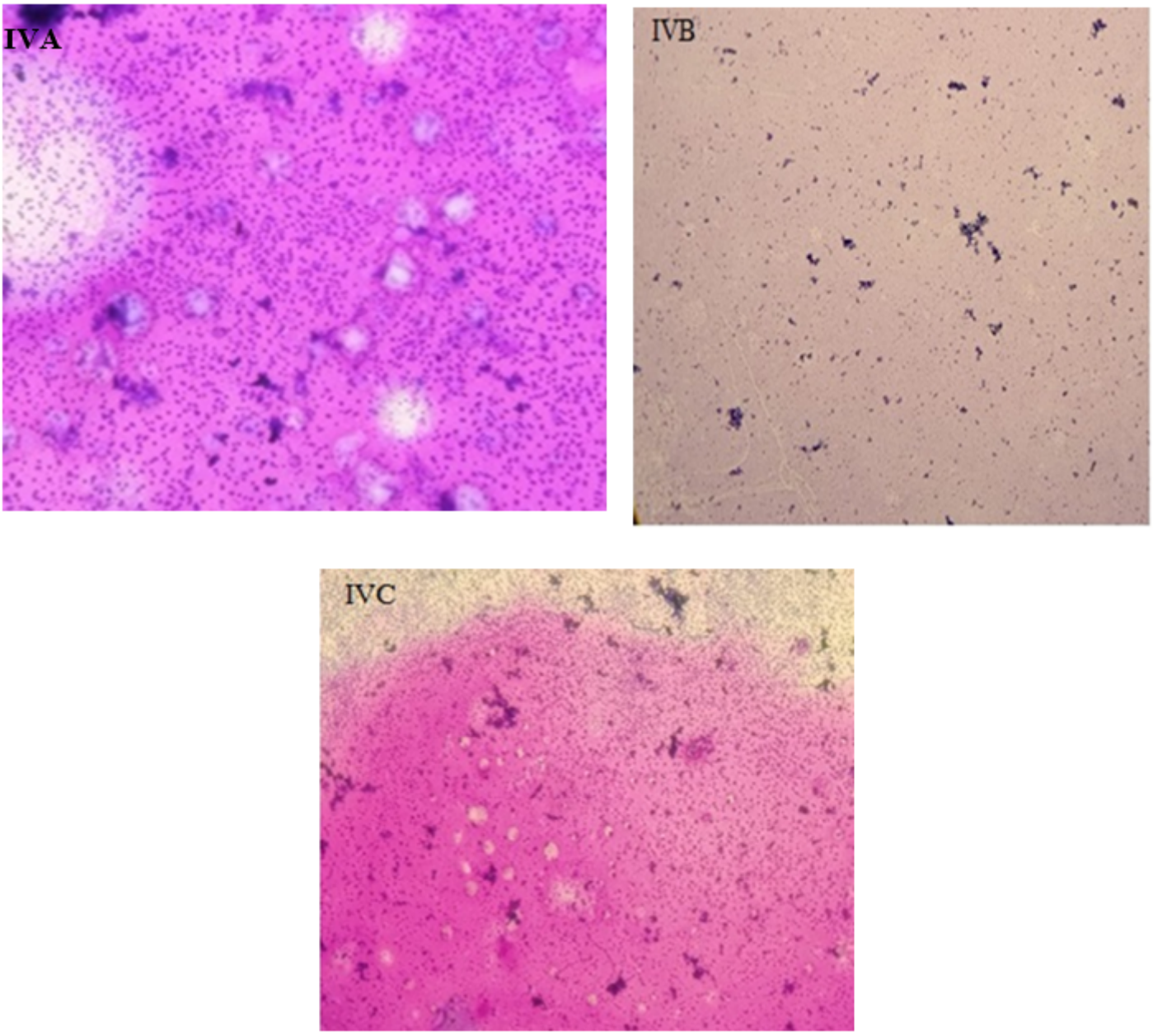

Figure 2

Peripheral blood smears for platelet adhesion analysis of IVA, IVB and IVC at 400× magnification

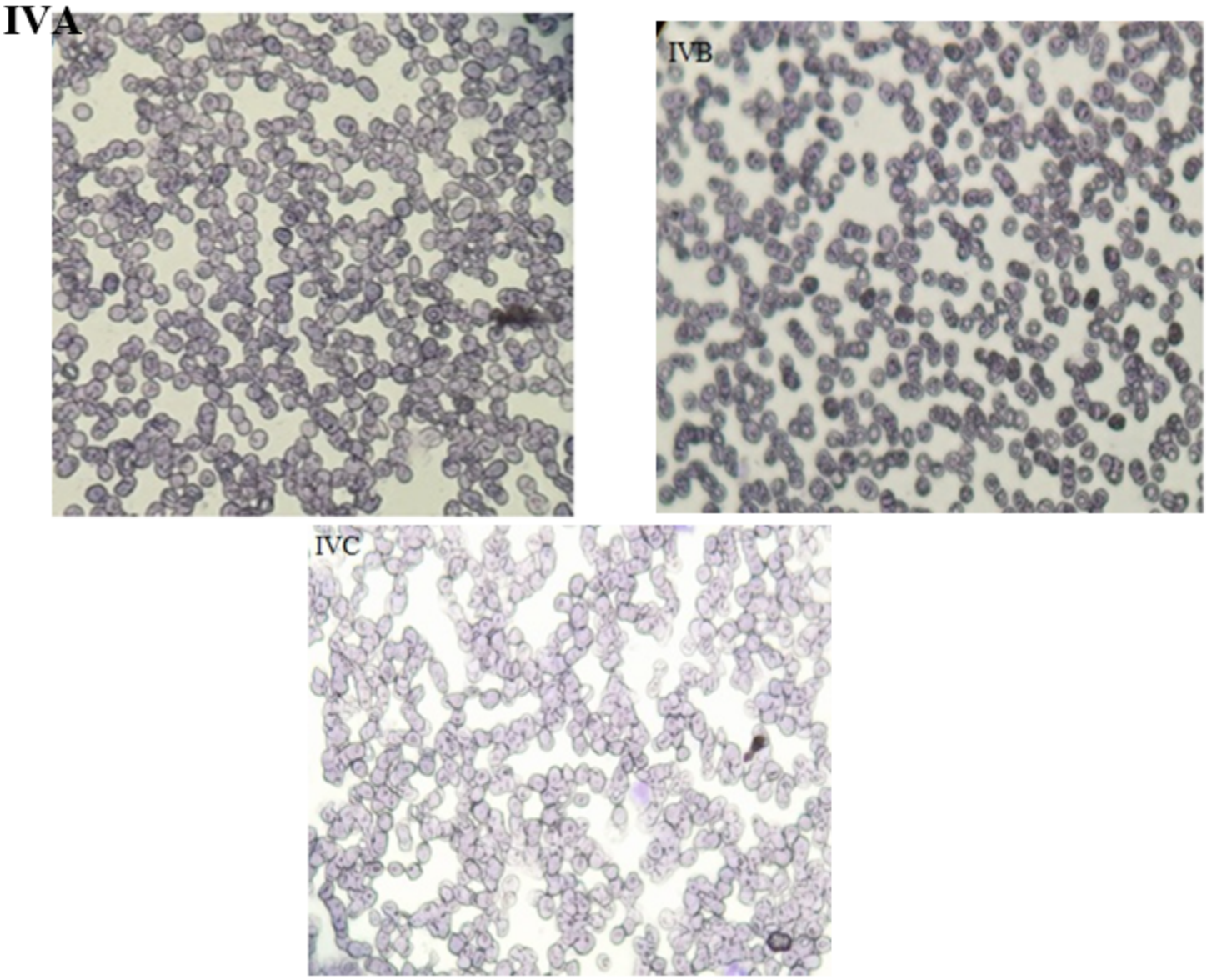

Figure 3

Wright-Giemsa Stains IVA, IVB and IVC at 400× magnification for erythrocyte morphology analysis

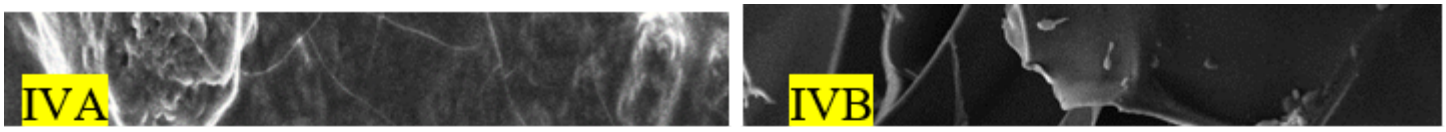

Figure 4

SEM micrograph of sample IVA, IVB and IVC 200µm

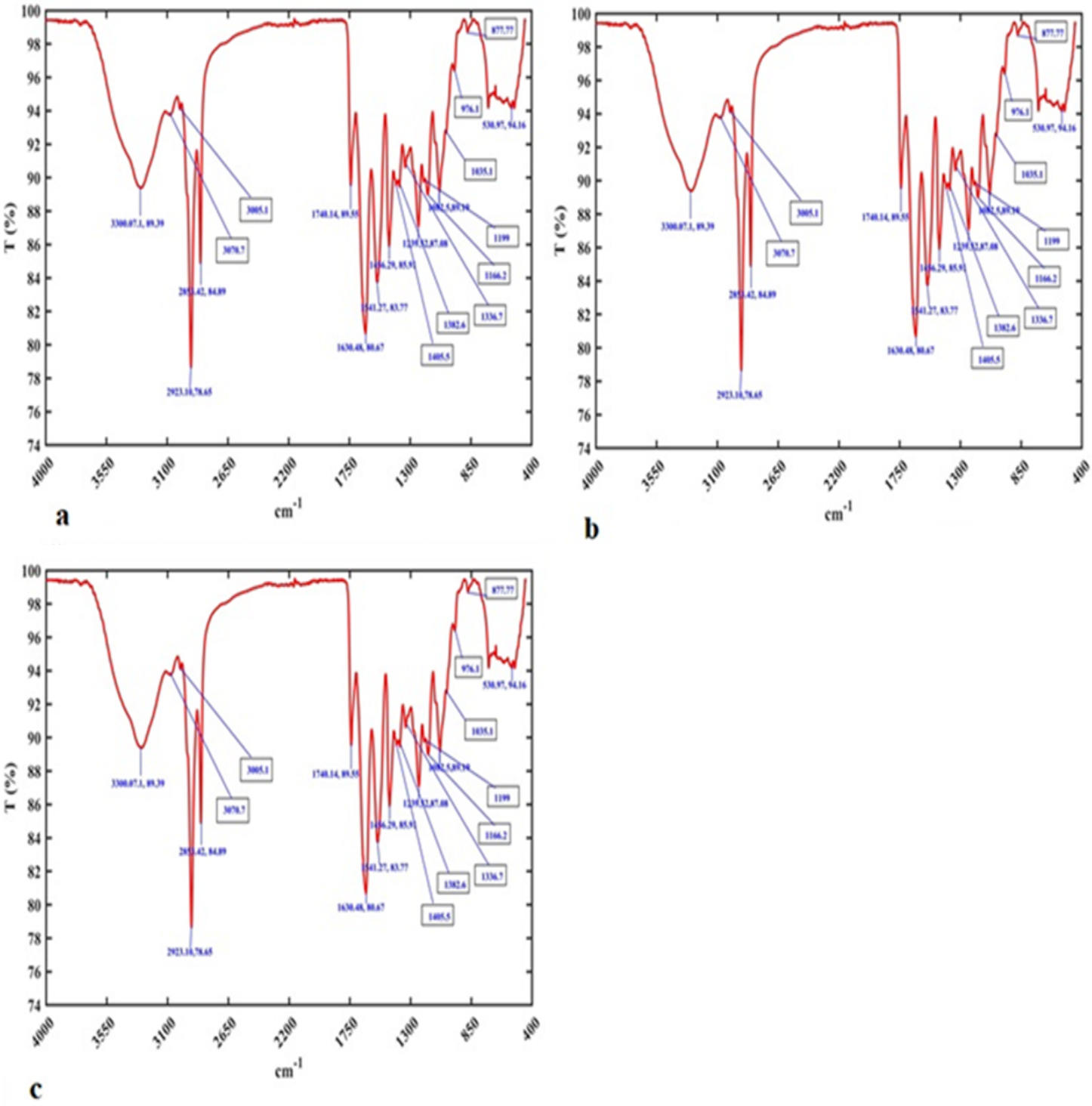

Figure 5

FTIR spectra of sample (a) IVA, (b) IVB and (c) IVC) Hydrogels