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Radiolabelling and cellular binding studies of metformin and indomethacin loaded dual niosome formulations on human colorectal adenocarcinoma cell line

Meliha EKINCI [1]*, Emine Esin CALISKAN [2], Burak CAKAR[3], Yalcin Celik AYDIN[2], Derya ILEM-OZDEMIR[1], Yigit UYANIKGIL[3,4,5], Emel Oyku CETIN UYANIKGIL[2]

1 Department of Radiopharmacy, Faculty of Pharmacy, Ege University, Izmir, Turkey.
2 Department of Pharmaceutical Technology, Department of Biopharmaceutics and Pharmacokinetics, Faculty of Pharmacy, Ege University, Izmir, Turkey.
3 Department of Histology and Embryology, Faculty of Medicine, Ege University, Izmir, Turkey.
4 Department of Stem Cell, Health Science Institute, Ege University, Izmir, Turkey.
5 Cord Blood, Cell and Tissue Research and Application Centre, Ege University, Izmir, Turkey.
* Corresponding Author. E-mail: melihaekinci90@gmail.com (M.E.); Tel. +90-232-311 32 82.

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ABSTRACT: In the current work, film hydration was used for producing indomethacin- and metformin-loaded dual niosomes with a particle size of < 150 nm, a polydispersity index of < 0.5, and a charge of between -30 and -50 mV. [^{99m}Tc]Tc was used as a radiolabel for all niosome formulations using stannous chloride as reducing agents. Radioactive thin-layer chromatography (RTLC) was used to evaluate the niosome formulations' radiochemical purity (RP) and stability. Next, the cellular binding of reduced/hydrolyzed (R/H)-[^{99m}Tc]$NaTcO_4$ and [^{99m}Tc]Tc-labelled niosome formulations was evaluated in human colorectal adenocarcinoma cells. Radiolabelling all niosomes with [^{99m}Tc]Tc for 15 min at 500 µg.mL^{-1} stannous chloride resulted in successful radiolabelling, with an observed RP of over 95%. The cell binding percentages of [^{99m}Tc]Tc-labelled niosome formulations in cancer cells were higher than those of R/H-[^{99m}Tc]$NaTcO_4$. Finally, the recently developed dual-niosome formulations of metformin and indomethacin labelled with [^{99m}Tc]Tc may prove effective for nuclear medicine imaging.

KEYWORDS: Niosome; technetium-99m; radiolabelling; cell culture.

1. INTRODUCTION

One of the main reasons of death for a long time has been cancer [1,2]. Early identification and successful cancer therapy lowers the risk of death and morbidity, as well as the expense and duration of treatment. Non-invasive functional data at the cellular levels can be obtained by nuclear medicine imaging. Nuclear imaging techniques are often used to detect abnormalities at very early stages [3]. In this field, technetium-99m ([^{99m}Tc]Tc) is a widely used radioisotope for radiolabelling because of its short half-life of 6 h, flexible chemistry, and low radiation dose, which produces gamma energy of 140 KeV [4].

Indomethacin, nonsteroidal anti-inflammatory drug (NSAID), is used to treat inflammation. Studies have shown that NSAIDs reduce the risk of colorectal cancer by 40-60% [5]. Metformin is an antidiabetic agent used in the treatment of type 2 diabetes. On cancer cells, metformin has been demonstrated to have anti-inflammatory properties. It reduces cancer cell growth and proliferation through its direct effects on cancer cells and indirect effects on reducing endogenous hyperinsulinemia [6].

Niosomes are self-assembling spherical bilayer vesicles made of hydrated nonionic surfactant monomers [7]. They are effective nanocarriers for delivery of chemicals, vaccines, genes, anticancer agents, and proteins. They have several important advantages, including affordability, increased stability, and ease and longer of storage [8].

The purpose of this work was to examine the potential use of radiolabelled niosome formulations as nanocarriers for cancer diagnostics. The film hydration process was employed for producing niosome formulations, and developed niosomes were characterized according to size distribution (polydispersity

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index (PDI)), particle size, zeta potential, image profile, and entrapment efficiency. The niosome formulations were radiolabelled with [^{99m}Tc]Tc, and then, they were tested *via* radioactive thin-layer chromatography (RTLC) quality control procedures. The *in vitro* stability of the radiolabelled niosomes in various media was investigated. Subsequently, an *in vitro* comparative study was carried out using HT-29 cell lines (a type of human colorectal cancer) to evaluate the potential utility of radiolabelled niosome formulations in nuclear imaging, in comparison to reduced/hydrolyzed (R/H)-[^{99m}Tc]$NaTcO_4$.

2. RESULTS AND DISCUSSION

2.1. Preparation and Characterization of Niosome Formulations

The film hydration technique was effectively used in the present study to produce all of the niosome formulations [9,10]. The characterization study findings were presented in Table 1.

Table 1. Characterization study results.

Formulations	PS (nm)	PDI	ZP (mV)	EE (%)
Blank niosomes	130.5 ± 3.231	0.255 ± 0.031	-38.22 ± 0.057	-
Indomethacin niosomes	132.8 ± 1.563	0.423 ± 0.126	-40.51 ± 1.631	66.12 ± 0.1
Metformin niosomes	142.6 ± 2.781	0.364 ± 0.254	-39.36 ± 1.236	66.35 ± 0.2
Dual niosomes	136.4 ± 2.055	0.423 ± 0.158	-36.26 ± 0.573	54.23 ± 0.1 (İ) 53.01 ± 0.3 (M)

***PS:** Mean particle size, **PDI:** Mean polydispersity index, **ZP:** Mean zeta potential value, **EE%:** Entrapment Efficiency, **İ:** İndomethacin, **M:** Metformin.

The obtained findings indicate that the niosome formulations have been produced with a PDI value between 0.255 ± 0.031 and 0.423 ± 0.158, and a particle size between 130.5 ± 3.231 nm and 142.6 ± 2.781 nm. The process for producing niosome formulations proved to be simple as well as repeatable [11].

The physicochemical characteristics of niosome formulations play a key role in determining their performance *in vivo.* For intravenous administration, a well-constructed niosome formulations should have a restricted PDI in the nanometer range. In order to guarantee the stability for injectable nanosized formulations, nanocarriers with a mean diameter of less than 200 nm have been proposed [12]. Regarding vesicular system applications for drug delivery, a PDI of < 0.5 is frequently regarded as suitable and indicates a homogenous dispersion of drug delivery systems [13]. In this work, sonication was employed to reduce the niosome formulations' particle size and PDI value [14]. Our findings indicate that the produced niosomal dispersions are appropriate for intravenous drug delivery applications.

The interaction between the biological system and the formulation is influenced by the zeta potential value. Zeta potentials of < −50 mV or > +50 mV can decrease particle aggregation in comparison to uncharged particles [15]. In the current work, negatively charged niosomes had zeta potential values of less than -50 mV.

Scanning electron microscopy (SEM) pictures of the niosome formulations were shown in Figure 1.

According to SEM images, all niosome formulations were nearly spherical, and the results were compatible with the Malvern ZetaSizer measurements.

2.2. Radiolabelling, Radiochemical Purity and Stability of Niosome Formulations

The direct radiolabelling approach was used in this study to radiolabel niosome formulations using the [^{99m}Tc]Tc, which the reducing agent decreased to lower oxidation states. Based on our previous study, stannous chloride (500 µg.mL^{-1}) was selected as the reductant agent [11]. The radiochemical purity (RP) (%) values of all formulations were shown in Figure 2.

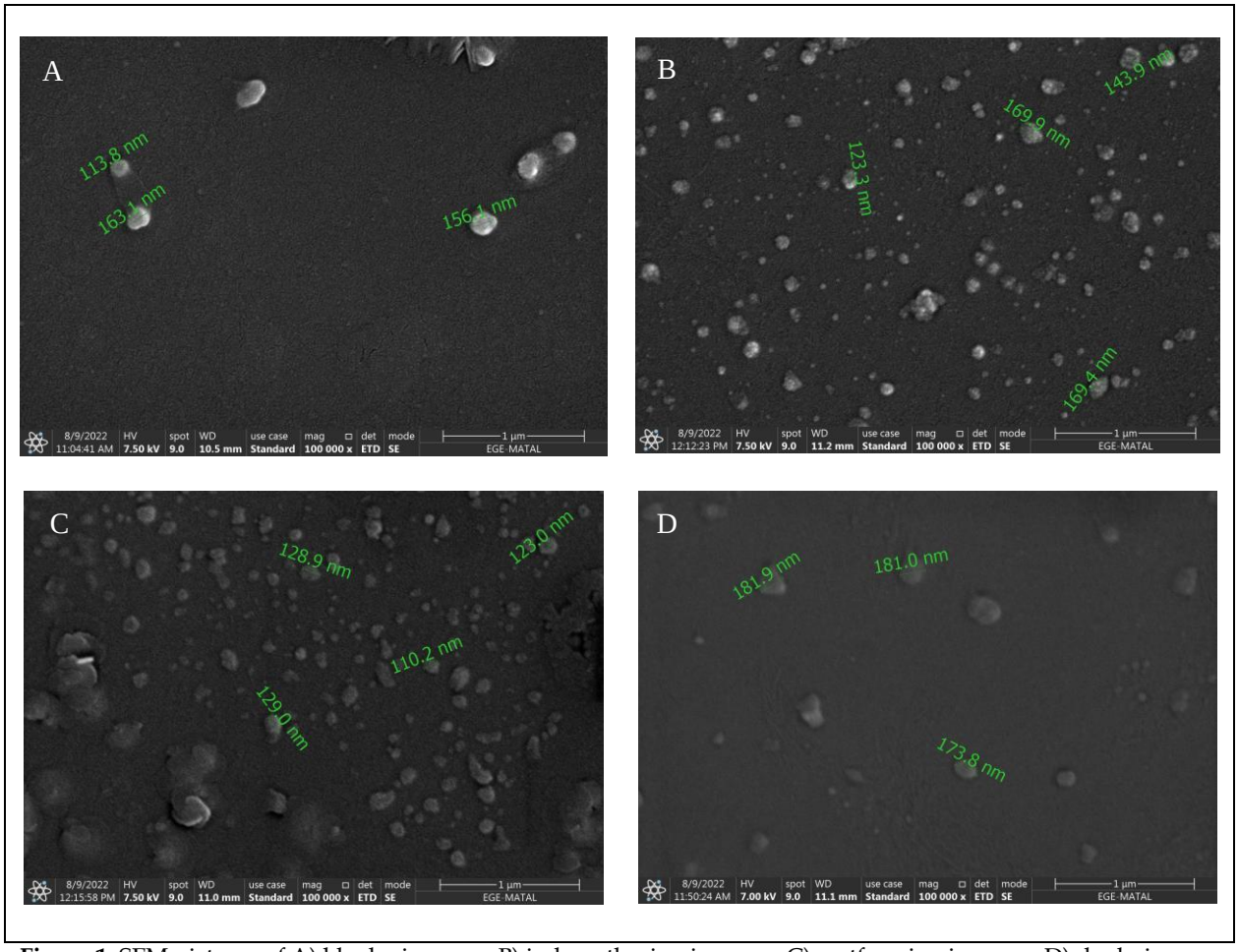

Figure 1. SEM pictures of A) blank niosomes, B) indomethacin niosomes, C) metformin niosomes, D) dual niosomes.
***SEM:** Scanning electron microscopy.

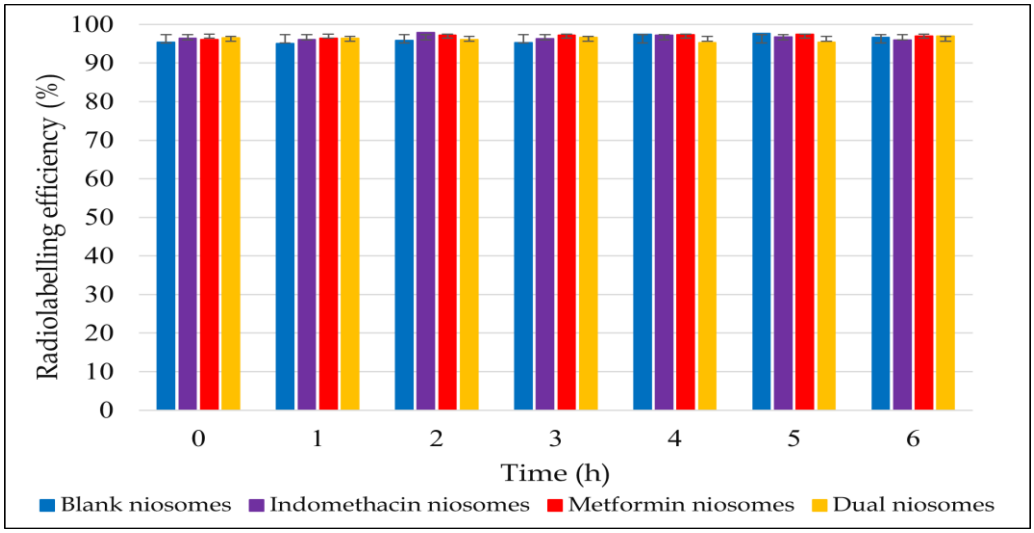

Figure 2. Radiolabelling efficiency of niosome formulations.

According to the obtained results, the RP of all radiolabelled niosome formulations was over 95%. RTLC was used for quality control of the radiolabelled niosome formulations. For RTLC, whereas [^{99m}Tc]Tc-niosomes and radiocolloids stayed at the origin, the percentage of [^{99m}Tc]$NaTcO_4$ that migrated with the solvent front was ascertained using saline (SF) as the mobile phase. While [^{99m}Tc]Tc-niosomes and [^{99m}Tc]$NaTcO_4$ migrated to the solvent front, the % of radiocolloids that stayed at the origin was determined using another developing solvent containing the Pyridine/Acetic acid/Water (PAW) solution (3:5:1.5).

Figure 3 displays the RTLC chromatograms of the [^{99m}Tc]Tc-niosomes obtained using these developing systems. The RP of [^{99m}Tc]Tc-labelled niosome formulations was more than 95% in these conditions.

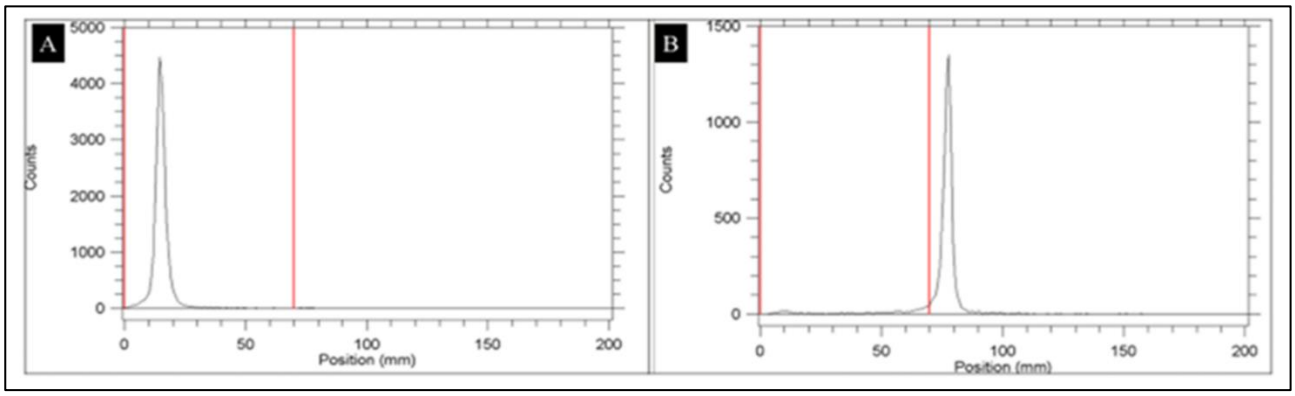

Figure 3. RTLC chromatograms of [^{99m}Tc]Tc-labelled niosome formulations in various mobile phases: (A) Saline, (B) Pyridine/Acetic acid (100%)/Distilled water (3:5:1.5).

The formulations of [^{99m}Tc]Tc-labelled niosomes were evaluated for stability in serum (fetal bovine serum (FBS): phosphate buffer solution (PBS) (1:1)), cell medium at 37 °C, and SF at 25 °C (Figure 4).

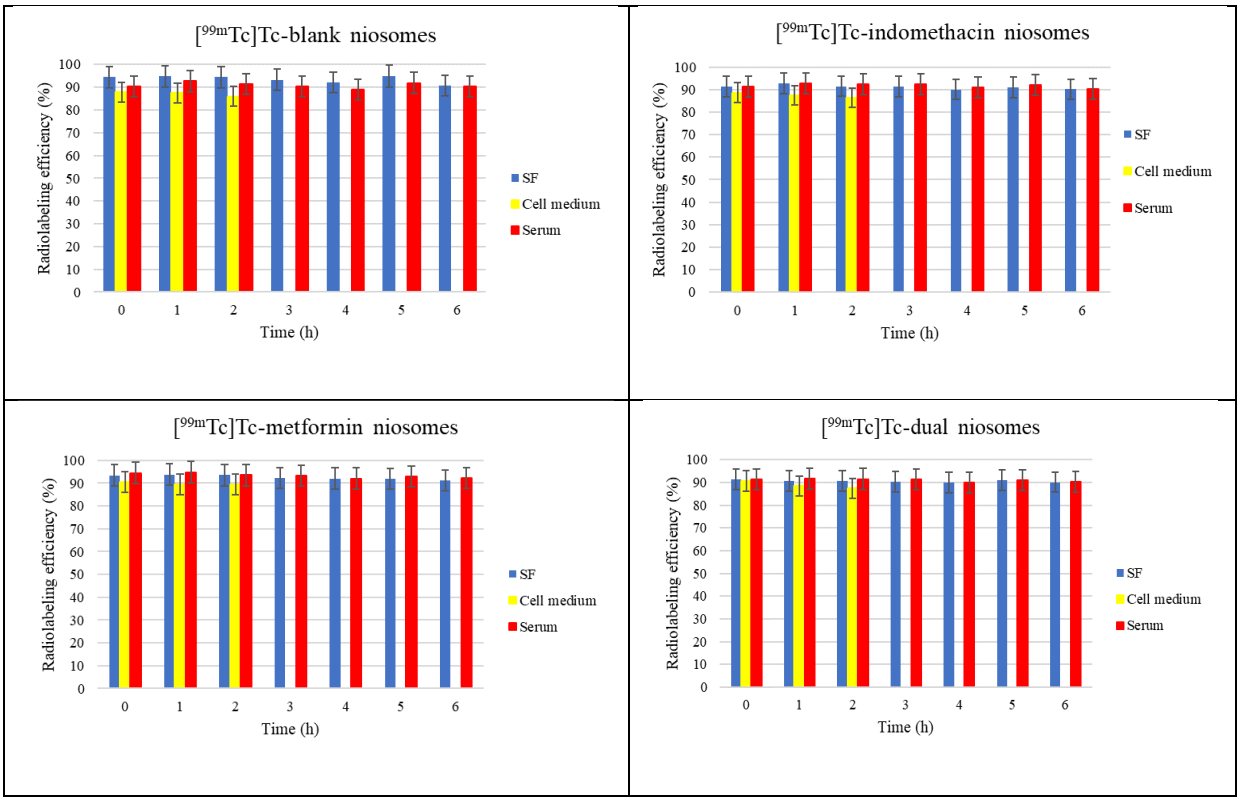

Figure 4. The stability of [^{99m}Tc]Tc-labelled niosome formulations in serum, SF, and cell medium.
***SF:** Saline, **Cell medium:** McCoy's 5A supplemented with 10% fetal bovine serum.

The stability results showed that all formulations of [^{99m}Tc]Tc-labelled niosomes were stable in various media (RP ≥ 90%), including serum, cell medium, and SF.

2.3. Cellular Binding of Radiolabelled Niosome Formulations

The current study investigated into the radiolabelled niosome formulations' capacity to bind HT-29 cells. The available half-life of [^{99m}Tc]Tc allowed for the tests to be performed for 2 hours. **Figure 5** displays the percentage of cellular binding of [^{99m}Tc]Tc-labelled niosome formulations into HT-29 cell lines along with R/H-[^{99m}Tc]$NaTcO_4$ (as a control group).

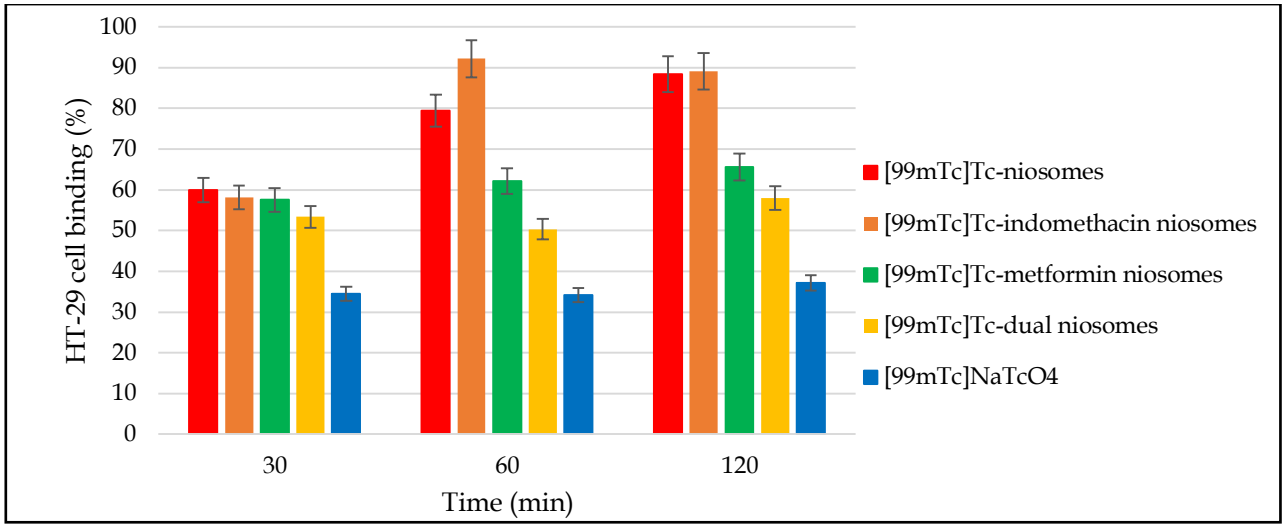

Figure 5. Ability of [^{99m}Tc]Tc labelled niosome formulations and R/H-[^{99m}Tc]$NaTcO_4$ to bind HT-29 cell line.

As seen in Figure 5, the [^{99m}Tc]Tc-labelled niosome formulations had greater HT-29 cellular binding activity than control group during the experiment. The cellular binding percentage of the [^{99m}Tc]Tc-blank niosome formulations ranged from 59.92 ± 2.43% to 88.45 ± 3.54%. The binding of [^{99m}Tc]Tc-indomethacin niosome formulations was higher than that of all other radiolabelled niosome formulations at 60 minutes (92.20 ± 2.56%). Also, the cell binding percentage of [^{99m}Tc]Tc-dual niosome formulations ranged from 50.31 ± 3.15% to 58.00 ± 1.58% during experimental time.

Additionally, the cell binding percentage of R/H-[^{99m}Tc]$NaTcO_4$ varied from 34.43 ± 1.56% at 30 minutes to 37.14 ± 1.78% at 120 minutes in order to test the study. This result confirms the high RP and stability of our radiolabelled niosome formulations and shows that they behaved differently in cell media than R/H-[^{99m}Tc]$NaTcO_4$.

3. CONCLUSION

In conclusion, it should be noted that [^{99m}Tc]Tc-niosomes were efficiently produced as promising nanocarriers in nuclear medicine. The obtained results of the radiolabelling study showed that the optimal reducing agent needed to radiolabel niosomes was stannous chloride (500 µg.mL^{-1}). Using RTLC, it was shown that the RP of [^{99m}Tc]Tc labelled all niosome formulations was higher than 95%. The radiolabelled niosomes remained stable in serum, SF, and cell medium for up to 6 hours. HT-29 cell lines had greater [^{99m}Tc]Tc-niosome binding percentages than R/H-[^{99m}Tc]$NaTcO_4$. Consequently, the recently produced [^{99m}Tc]Tc-dual niosomes are good candidates for prospective *in vivo* applications in nuclear imaging down the line.

4. MATERIALS AND METHODS

4.1. Materials

Stannous chloride, Tween 60, Span 60, indomethacin, metformin, and cholesterol were supplied from Sigma-Aldrich. [^{99m}Tc]$NaTcO_4$ was eluted using a [^{99}Mo]Mo/[^{99m}Tc]Tc generator (Ege University, Turkey). All chemicals and solvents were all supplied by Merck. The human colorectal adenocarcinoma cells (HT-29 cell line) were obtained from ATCC. All materials and reagents used in cell culture study was purchased Gibco Invitrogen.

4.2. Preparation of Niosome Formulations

Niosome formulations (blank niosomes, indomethacin niosomes, metformin niosomes, and dual niosomes) were formed by the process of film hydration [9-11]. In short, cholesterol, surfactants (Span 60 and Tween 60) [1:1 molar ratio], and the active ingredients (indomethacin and metformin) were dissolved in 10 mL of chloroform. The chloroform was evaporated in a rotary evaporator at 60 °C and 100 rpm. The resultant films were hydrated with phosphate buffer (pH 7.4) for one hour at 100 rpm and 60 °C. Sonication at 350 W for 30 s cycles was then used to reduce the particle size for 15 minutes. Three times, the niosomes were precipitated using ultracentrifugation at 20,000 rpm for one hour. After adding 3% trehalose solution to

the niosomes, the system was finally frozen at -80 °C and lyophilized using a freeze-dryer equipment for 48 hours [16].

4.3. Characterization of Niosome Formulations

4.3.1. Particle Size and Distribution Value

The mean particle size and distribution of the niosomes were determined by Malvern Zetasizer Nano ZS. The analysis was carried out at 25 °C and a detector angle of 173°.

4.3.2. Zeta Potential Value

Using a folded capillary cell (DTS1070), the zeta potential value of the niosomes was evaluated by Malvern Zetasizer Nano ZS. Zeta potential values are presented as mV.

4.3.3. Drug Entrapment Efficiency

To separate the niosomes from the non-entrapped drugs, niosome formulations were centrifuged for one hour at 4°C and 20,000 rpm. Using High-performance liquid chromatography (HPLC), a measurement was carried out of the free drug content in the supernatant. To guarantee that all traces of the free drug were removed, this procedure was carried out three times. The niosomes' drug entrapment % was computed [17].

4.3.4. Scanning Electron Microscope Image

The image profile of the niosome formulations was examined using SEM. In order to do this, dried niosomes were coated with gold using an evaporator (EMITECH K550X Sputter Coater) for 1.5 minutes at 15 mA and 6 × 10^{-2} mbar, with a thickness of 10 nm. After that, the coated formulations were scanned at 7.5 kV and 100,000× magnification using a Philips XL-30S FEG SEM [18].

4.4. Radiolabelling of Niosome Formulations

Using 500 µg.mL^{-1} of stannous chloride (in bidistilled water), all niosomes were radiolabelled with [^{99m}Tc]Tc [11,19,20]. SF solution was used to elute [^{99m}Tc]$NaTcO_4$ from the [^{99}Mo]Mo/[^{99m}Tc]Tc generator. In short, a stannous chloride solution was combined with 0.1 mL of [^{99m}Tc]Tc (37 MBq.mL^{-1}). A 1 mL of niosomal suspension was added, vortexed for 1 minute, and then allowed to incubate for 15 minutes. RTLC was used to evaluate the niosomes' radiolabelling efficacies.

4.4.1. Radiochemical Purity of Niosome Formulations

For six hours, the RP of the radiolabelled niosomes was assessed by RTLC at various intervals. Whatman No. 3 paper and instant TLC–silica gel–coated papers (ITLC-SG) used as the stationary phases, while SF and PAW (3:5:1.5) solvent mixtures were utilized as the mobile phases [11]. RP (%) was calculated using the **Equation 1**:

RP (%) = [100-(free [^{99m}Tc]Tc (%)+colloidal [^{99m}Tc]Tc (%))] (1)

4.4.2. Stability of Radiolabelled Niosome Formulations

The radiolabelled niosomes' stability was assessed in serum at 37 °C, and culture media (McCoy's 5A) at 37 °C, and SF at 25 °C. To do this, 400 µL of serum, SF, and cell media were mixed with 100 µL of radiolabelled niosome formulations. To assess the stability of radiolabelling, RTLC analysis was performed on the samples.

4.5. Cell Culture Study

At 37 °C in a 95% humidified atmosphere with 5% CO_2, HT-29 cells were cultured in McCoy's 5A medium supplemented with 10% FBS and 0.5 mg.mL^{-1} L-glutamine/penicillin. After being grown in 75 cm^2 flasks until they achieved 90% confluence, 1 × 10^6 HT-29 cells were placed at a density of one per well in plates.

4.5.1. Cellular Binding of Radiolabelled Niosomes

Cell binding tests were performed on HT-29 cells using radiolabelled niosomes and R/H-[^{99m}Tc]$NaTcO_4$ (as a control). In order to do this, the cells were incubated up to 2 h in an incubator with [^{99m}Tc]Tc-labelled niosomal formulations and [^{99m}Tc]$NaTcO_4$, which included 18.5 MBq of radioactivity. The

culture media had been collected in a centrifuge tube at the end of the every incubation time. Afterwards, 0.5 mL of trypsin-EDTA was applied to the cells in order to collect them. Following one another, 0.5 mL of McCoy's 5A (0.5 mL) and PBS (pH 7.4) were used to wash the six-well plates in order to get rid of any weakly bound surface [^{99m}Tc]Tc radioactivity. For 5 minutes, the HT-29 cells were centrifuged at 3000 rpm. Then, the cells were transferred to a second tube, and the supernatant was poured into the initial tube. Using a gamma counter, the [^{99m}Tc]Tc radioactivity in the HT-29 cells and supernatant was determined. The **Equation 2** was used to determine the percentage of radiolabelled formulations that bound to cells:

HT-29 cell binding (%) = (radioactivity of cells/(radioactivity of cells + supernatant)) ×100 (2)

4.6. Statistical Analysis

Microsoft Excel was used for the analysis of the findings. Every experiment was run through at least three times, and the differences within each group were represented by the standard deviation (SD). When the Student's t-test was used to compare the experimental groups statistically, p values of less than 0.05 were considered statistically significant.

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